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An autism-associated Mint2 mutation alters neurexin trafficking and synaptic function

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AN AUTISM-ASSOCIATED MINT2 MUTATION ALTERS NEUREXIN TRAFFICKING AND SYNAPTIC FUNCTION

by

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I think this sum up this incredible journey quite well. Don't you agree?!

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ABSTRACT

Autism spectrum disorders (ASD) comprise a heterogeneous group of neurodevelopmental disorders characterized by complex genetic etiology. Mutations in human *APBA2*, which encodes for the neuronal adaptor protein Mint2, have been genetically linked to ASD patients. *APBA2* maps to the distal portion of chromosome 15q13.1, a region commonly deleted in Prader-Willi and Angelman neurodevelopmental disorders and duplicated in cases of autism, making *APBA2* an attractive candidate gene associated with autism. Seven novel nonsynonymous coding variants in *APBA2* in ASD subjects have been identified, five of which were predicted to affect protein function; however, they have not been examined functionally. Mint2 belongs to a family of neuronal adaptor proteins that are important for synaptic function. Mint2 interacts directly with the cell adhesion protein neurexin-1α, as part of a multi-protein complex that acts as a facilitator of neurotransmitter release. Together, these data suggest that Mint2 plays an important role in neuronal function, and sequence variations in Mint2 may alter neuronal dysfunction associated with ASD.

This thesis examines a point mutation in Mint2, which changes a conserved asparagine residue to a serine (N723S) in the second PDZ domain of Mint2, which binds

to neurexin-1α. We found the Mint2 N723S mutation did not affect the binding to neurexin-1α; however, it dramatically altered neurexin-1α stabilization and trafficking in HEK293T cells. While Mint2 wild type greatly increased neurexin-1α at the membrane, Mint2 N723S showed a decreased membrane level of neurexin-1α, indicating the steady-state surface expression of neurexin is affected by the Mint2 N723S mutation. Also, we found that Mint2 N723S decreased neurexin localization in axons and the presynaptic terminal in neurons, which correlated with a decrease in synaptogenesis and miniature event frequency in excitatory synapses in neurons. Together, these results suggest that Mint2 N723S leads to dysfunction in neuronal development, in part due to alterations in intracellular neurexin trafficking and altered synaptic function of Mint2, as potential mechanisms that contribute to ASD pathogenesis.

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LIST OF ABBREVIATIONS

AD ..... Alzheimer's disease

AMPA ..... α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

APBA ..... Amyloid beta A4 precursor protein-binding family A member

APP ..... Amyloid precursor protein

ASDs ..... Autism spectrum disorders

ATP ..... Adenosine triphosphate

BIFC ..... Bimolecular fluorescence complementation

C ..... Celsius

CASK ..... calcium/calmodulin dependent serine protein kinase

CID ..... Cask interacting domain

CNV ..... Copy number variant

COS7 ..... African Green Monkey SV40-transformed kidney fibroblast cell line

C-terminal ..... Carboxyl terminal

DAPI ..... 4',6-diamidino-2-2phenylindole

DDM ..... n-dodecyl-β-D-maltoside

DIV ..... Days *in vitro*

DMEM ..... Dulbecco's modified eagle medium

DNA ..... Deoxyribonucleic acid

ECL ..... Enhanced chemiluminescence

EDTA ..... Ethylenediaminetetraacetic acid

EGF ..... Epidermal growth factor

EGTA ..... ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid

ER ..... Endometiral reticulum

FRAP ..... Fluorescence recovery after photobleaching

GABA ..... γ-Aminobutyric acid

GAPDH..... Glyceraldehyde-3-phosphate dehydrogenase

GFP .....Green fluorescent protein

GM130 ..... Golgi marker 130 kDa

GPCR .....G-protein-coupled receptor

GST .....Glutathione *S*-transferase

HEK293T .....Human endothelial kidney-293T cell line

ICC..... Immunocytochemistry

kDa..... Kilodalton

KIF ..... Kinesin-like protein

KO.....Knockout

LNS ..... Laminin/neurexin/sex hormone-binding globulin

M .....Molarity

mEPSC..... Miniature excitatory postsynaptic current

MID.....Munc18 interacting domain

Mint.....Munc18 interacting protein (synonymous with APBA)

Munc18 .....Mammalian uncoordinated-18

NLGN ..... Neuroligin

NRXN .....Neurexin

N-terminal ..... Amino terminal

PAGE ..... Polyacrylamide gel electrophoresis

PBS ..... Phosphate buffered saline

PCR ..... Polymerase chain reaction

PDZ ..... PSD-95/Disc Large/Zonus-Occludens-I

PFA ..... Paraformaldehyde

PSD95 ..... Postsynaptic density protein 95

PTB ..... Phosphotyrosine binding domain

RNA ..... Ribonucleic acid

SDS ..... Sodium dodecyl sulfate

SEM ..... Standard error of the mean

SNP ..... Single-nucleotide polymorphism

TBST ..... Tris buffered saline with Tween 20

TTX ..... Tetrodotoxin

WB ..... Western blotting

CHAPTER ONE: Introduction

Autism spectrum disorder (ASD) overview

Autism spectrum disorder (ASD) is a heterogenous neurodevelopmental disorder with broad pathological and unknown neurobiological etiology. Autism is diagnosed based on three behavioral criteria, namely language impairment, social deficit, and repetitive behavior, suggesting that each behavioral abnormality may result from different genes associated with different areas of the brain (Happe *et al.* 2006). Although autism is diagnosed strictly on behavior criteria, other comorbidities, such as mental retardation, epilepsy, sleep disorder, and anxiety disorder are often diagnosed in autistic individuals, suggesting similar brain dysfunctions are involved in these disorders (Herbert 2005).

Autism has a broad spectrum of clinical manifestations and striking interpersonal differences between affected individuals. Based on studies of thousands of children, the field has compiled extensive clinical diagnostic criteria, but the corresponding neuropathological diagnostic criteria are lagging behind, partially due to a lack of availability of post-mortem brains. The present studies show that the most consistent neuroanatomical abnormality in autism is a usually large brain size and a large contribution of white matter to the increase in brain volume (Herbert 2005).

Epidemiological studies indicate a dramatic rise in autism cases in the recent years (Herbert 2005). Before 1990, autism was considered a rare disorder, affecting 3-4 in 10,000 individuals. Currently, autism affects around 3 to 6 in 1000 children and mostly males (Muhle *et al.* 2004), but the gender preference of the disorder is not well

understood. The increased frequency is probably due to a combination of more public awareness and broadened diagnostic criteria. The onset of symptoms starts around three years of age, and affected individuals usually require lifelong assistance (Fombonne 2005).

Genetic association of autism

The cause of autism is not clear, but genetics certainly play a major role. Autism is the one of the most heritable neurodevelopmental disorder based on twin studies (Folstein and Rosen-Sheidley 2001). The concordance rate of autism for monozygotic twins is 70% as compared to 5% in dizygotic twins (Sebat *et al.* 2007), suggesting that genetics contribute a major role in the etiology of autism. However, the mode of inheritance is not well known.

Despite the strong genetic correlation, not a single gene to date has been identified to contribute to a significant number of autism cases, suggesting that other factors are involved. The causes of autism are likely the results from a complex interplay of genetics, environment, and experience. Environmental factors such as exposure to teratogens, prenatal viral infections, such as rubella and cytomegalovirus during and after pregnancy have been associated with some cases of autism (Muhle *et al.* 2004).

Autism displays a pronounced degree of genetic heterogeneity, which presents a major obstacle to research. Based on linkage studies to identify candidate genes, many loci on 20 different chromosomes have been identified to be associated with autism (Klauck 2006). Regions confirmed by multiple linkage studies are on chromosome 1p,

5q, 7q, 15q, 16p, 17q, 19p, and Xq (Klauck 2006), which is consistent with the theory that many genes are involved and likely leading to common neurodevelopmental pathways. Also, cytogenetic abnormalities in various loci on all chromosomes have been identified in more than 5% of affected children (Vorstman *et al.* 2006). However, both linkage and cytogenetic studies fall short of validating the exact genes that are involved in autism, with the exception of some rare syndromic forms of autism, such as Rett syndrome, where a single gene has been unambiguously identified to cause autism.

Based on recent protein interaction networks, autism candidate genes converge to three common pathways; synaptic function, chromatin remodeling, and WNT signaling (Iossifov *et al.* 2014)—see Fig. 1.1. The synaptic function pathway involves proteins that are essential for synapse function. Based on post-mortem brain studies from autism patients, no gross anatomical abnormalities were observed in the brain regions that are associated with autistic phenotypes. Therefore, it is more likely that smaller computational units such as individual synapses are at fault. This observation leads to the synaptic function pathway. Various proteins in the synaptic function pathway, such as NMDA receptor subunits, CASK, neuroligins, and neurexins have been linked to autism (Glessner *et al.* 2009). Another study found that genes, such as APBA2, CNTNAP1, and CAMK2B are regulated together in autism (Voineagu *et al.* 2011)—see Fig. 1.2. These genes are involved in synaptic function, vesicular transport, and neuronal projection, suggesting that a complex interaction among genes from multiple pathways are involved in autism.

Proteins in the chromatin-remodeling pathway are involved in transcriptional regulation and can alter virtually any neuronal function (Fig. 1.1). Similarly, the WNT signaling pathway can have wide ranging effects as it plays important roles in neuronal development and growth. The interactions are complex. Not only proteins within a common pathway can interact with each other, but proteins between independent pathways can also interact. For example, CTNNB1 (beta-catenin), an important protein that regulates neuronal development and growth in the WNT pathway, interacts with CHD8 (Chromdomain helicase DNA binding protein 8) in the chromatin remodeling pathway. This interaction further illustrates that autism is not a single-gene disease as many proteins from multiple pathways can be involved.

Mint family of adaptor proteins

Mints are multidomain adaptor proteins that assemble protein complexes for dynamic cellular processes in protein trafficking, plasticity, and signaling in neurons (Rogelj *et al.* 2006). Mammalians have three *MINT* genes (*MINT1-3* or *APBA1-3*), encoded on chromosome 9, 15, and 19, respectively(Okamoto and Südhof 1997, Okamoto and Südhof 1998). Mints are evolutionary conserved, for example, human and mouse Mint2 share over 90% sequence homology. Mint2 arises evolutionarilly later compared to Mint1, most likely due to gene duplication of Mint1 to provide redundant functions in the complex vertebrate brain.

Mints 1 and 2 are neuronal specific, while Mint 3 is ubiquitously expressed (Okamoto and Südhof 1997). Mint1 was first identified as a candidate gene for

Friedreich's ataxia (Duclos *et al.* 1993); however, this association was later proven to be false. Interest in Mints arose when it was found that all Mints bind to the amyloid precursor protein (APP) (Borg *et al.* 1996, Zhang *et al.* 1997), and misprocessing of APP contributes to the pathogenesis of Alzheimer's disease (AD). Because of the interaction between Mints and APP, the official gene name for Mint proteins are *APBA*1-3 (APP binding family A). Another group had independently identified Mints as Munc18-interacting proteins, because of their interaction with Munc18, a synaptic vesicle docking protein required for synaptic transmission (Okamoto and Südhof 1997). However, only Mints 1 and 2 can bind to Munc18-1 since Mint3 lacks the Munc18-interacting domain (MID) (Okamoto and Südhof 1998).

Structure of Mints

The Mint family of adaptor proteins are multidomain proteins that consists of a divergent amino (N)-terminus and conserved carboxyl (C)-terminus (Fig. 1.3). Mint1 is the only Mints containing the CASK-interacting domain (CID) on the N-terminus. Mint1, CASK, and Veli form a tripartite complex that couples presynaptic vesicle exocytosis to cell adhesion in neurons (Butz *et al.* 1998). Also in the N-terminus, neuronal Mints 1 and 2 binds to Munc18-1, an essential synaptic vesicle protein, linking Mints to synaptic vesicle exocytosis (Okamoto and Südhof 1997, Ho *et al.* 2006). This interaction is consistent with Mint knockout analyses, which revealed that Mints 1 and 2 are critical for presynaptic function (Ho *et al.* 2006, Ho *et al.* 2003). In the conserved C-terminus, the phosphotyrosine binding (PTB) domain of all three Mints binds to the

cytoplasmic tail of APP and alters its proteolytic processing which is centrally involved in the pathogenesis of Alzheimer's disease (Borg *et al.* 1996, Ho *et al.* 2008). Also, the PTB domain interacts with phosphatidylinositol biphosphate (PIP_2), suggesting that Mints are associated with the plasma membrane during vesicle docking before fusion (Okamoto and Südhof 1997). In the C-terminus, the two postsynaptic density-95/*Drosophila* discs-large/zona occludens-1 (PDZ) domains bind to various proteins *in vitro,* including neurexin I (Biederer and Südhof 2000), presenilin I (Lau *et al.* 2000, Biederer *et al.* 2002), and calcium channels (Maximov *et al.* 1999), which all are essential for synaptic functions. PDZ domains associate with other protein-binding motifs to form supramolecular complexes. The major function of PDZ-containing proteins is to act as scaffolds for multi-protein complex assembly. Structurally, PDZ domains recognize a free carboxylate group at the C-terminus of proteins (Sheng and Sala 2001). For example, the PDZ domains of neuronal Mints 1 and 2 recognize the C-terminus of neurexin I (Biederer and Südhof 2000). However, it is not known which PDZ domain directly interacts with neurexin I. While the sequences of PDZ domains between proteins are highly variable, the PDZ domains of Mints 1 and 2 are highly conserved, likely to account for Mints functional redundancy. Binding ligands of both PDZ domains of Mint1 were identified by target-assisted iterative screening (TAIS), a phage display-based approach (Swistowski *et al.* 2009). The binding histograms indicate that the recognition profiles between two PDZ domains are significantly different. The PDZ-2 is more promiscuous and has a high affinity for peptides containing a C-terminal valine.

Mints function is regulated by an autoinhibition domain in the C-terminus (Long *et al.* 2005, Matos *et al.* 2012, Xie *et al.* 2013). Mint1 binding to its targets is regulated by autoinhibition (Long *et al.* 2005). The highly conserved C-terminal tail of Mint1 folds back to the first PDZ domain, which inhibits target peptide binding. Our laboratory has shown that the C-terminal linker region of Mint1 folds back onto the PTB domain and modulates APP processing (Matos *et al.* 2012). Similarly, Mint2 has an autoinhibition domain that regulates APP binding with its open and close conformations (Xie *et al.* 2013).

The multi-domain nature of Mints allows the assembling of different protein complexes to mediate various neuronal functions (Fig. 1.4). Mint1 can form a tripartite complex with CASK and Munc18-1 (Biederer and Südhof 2000). Mint1 binding to Munc18-1 enhances its interaction with CASK. Mint2 can also interact with neurexin and Munc18-1 to form another tripartite complex at the presynaptic membrane. The functional importance of these interactions allows the assembling of different complexes for various neuronal functions.

Function of Mints

All single Mint knockout mice were viable and fertile (Ho *et al.* 2006). However, Mint1 knockout mice exhibited a significant reduction in body size (Ho *et al.* 2003), which was not observed in Mints 2 and 3 knockout mice (Ho *et al.* 2006). Mints 1 and 2 are involved in postnatal survival as 80% of Mints 1 and 2 double knockout mice died at birth. The surviving 20% mice have lower body weight and abnormal motor function

compared to wild type mice (Ho *et al.* 2006). Moreover, the surviving mice at 3-4 weeks of age exhibit decreased synaptic strength and a decline in presynaptic neurotransmitter release machinery (Ho *et al.* 2006).

Mints 1 and 2 have redundant domains and functions. However, they are not completely redundant based on the following three findings. First, *in situ* hybridization data identified that Mints 1 and 2 have different mRNA expression in adult murine brain (Nakajima *et al.* 2001), which suggests differences in tissue-specific promoters and/or tissue-specific mRNA degradation. Mint1 mRNA expression is enriched in the limbic system and Mint2 is enriched in the cerebral cortex, entorhinal cortex, and hippocampus (Nakajima *et al.* 2001).

Second, Mints 1 and 2 are differentially localized to inhibitory and excitatory neurons (Ho *et al.* 2003, Ho *et al.* 2006). Mint1 is involved in GABAergic synaptic transmission, and deletion of Mint1 in mice alters GABAergic synaptic transmission (Ho *et al.* 2003). Mint2 is involved in excitatory neurotransmission (Ho *et al.* 2006, Ho *et al.* 2008).

Third, Mints 1 and 2 have preferential intracellular localizations (Nakajima *et al.* 2001). Mint1 is localized to the somatodendrites and known for its postsynaptic function. A previous study has shown that Mint1 is localized in the dendrites to facilitate NMDA receptor trafficking to the postsynaptic membrane (Setou *et al.* 2000). In contrast, Mint2 is more involved in the presynaptic terminal. These different localizations suggest differences in binding partners and functions. However, this does not mean that Mints 1 and 2 have completely polarized localization in neurons. Numerous studies indicate that

Mints 1 and 2 are localized at both presynaptic and postsynaptic terminals and assume different functions depending on their location. Moreover, in vertebrate neurons, Mints 1 and 2 are concentrated at the Golgi apparatus (Biederer *et al.* 2002) and localized throughout axons and dendrites, strongly suggesting that Mints are involved in protein trafficking.

Mints 1 and 2 also have different temporal expression in the brain. Mint2 is expressed earlier in the brain compared to Mint1 indicating that Mint2 may be involved in early brain development compared to Mint1 (Ho *et al.* 2003). Behavioral studies indicate that Mint2 is involved in normal social development. Mint2 knockout mice displayed conflict avoidance and subordination in competitive feeding situations. This deficit in social interaction supports the role of Mint2 in normal emotional and social development (Sano *et al.* 2009).

Mutation in Mint2 links to autism

Autism is a neurodevelopmental disorder with a strong genetic component, including chromosomal aberrations. Among all the chromosomes, sex chromosomes and the long arm of chromosome 15 have been most frequently altered in autism (Gillberg 1998). The most often identified chromosomal abnormality is isodicentric chromosome 15 or idic(15), an inverted duplication of chromosome 15, in which the extra chromosomal material has been duplicated end-to-end like a mirror image (Fig. 1.5). The large extra genetic material accounts for some symptoms found in autism. It is important to note that idic(15) has some autistic-like characteristics, but it is not the same as autism.

In addition to such large-scale duplication, smaller intrachromosomal duplications of chromosome 15 have been reported in autism. In one family, maternal inheritance of 15q11-q13 duplication causes autism, but parental duplication of the same region leads to a normal phenotype, suggesting that the origin of duplication can be a contributing factor (Cook *et al.* 1997). In addition to duplication, microdeletion of chromosome 15q11-13 in Angelman syndrome causes intellectual disabilities and neurodevelopmental deficits. Angelman syndrome is a type of copy number variant with less gene dosage, but it has some autism-liked symptoms. In sum, this evidence strongly suggests that chromosome 15q11-13 must be tightly regulated as duplication or deletion of genetic material leads to autism and neurodevelopmental disorders.

The 15q11-q13 region spans several hundred thousand base pairs and consists of a handful of neuronal genes, including the *MINT2 (APBA2)* that resides within the chromosome region 15q13.1 and 15q13.3 (Fig. 1.5). Duplication and deletion of this region have been implicated in neurodevelopmental disorders (Cook *et al.* 1997, Babatz *et al.* 2009, Cook *et al.* 1998, Gillberg 1998, Schroer *et al.* 1998, Shao *et al.* 2002, Muhle *et al.* 2004, Christian *et al.* 2008)— see Fig. 1.5.

Mint2 copy number variants have been implicated in autism (Babatz *et al.* 2009)—see Fig.1.3. Copy number variants are the insertion or deletion of DNA fragments, typically from 50 base pairs up to several megabases. Insertion of DNA fragment would suggest more gene dosage and leads to protein overexpression. Deletion of DNA fragments would suggest less gene dosage and leads a partial knockout phenotype. In chromosome 15, the most common duplications are interstitial

dup15q11.2q13.1 and idic(15). Other less common copy number variants, such as duplication and deletion are also found in individuals affected with autism, schizophrenia, and mental retardation. It is important to point out that *MINT2* (*APBA2*), located in 15q13.1, is commonly duplicated or deleted in all above cases (Fig. 1.3), strongly suggest the importance of Mint2 in normal neuronal development.

In addition to copy number variants, seven novel non-synonymous sequence variants in Mint2 have been identified from 512 autism probands (Babatz *et al.* 2009). The functional consequences of these mutations have not been validated. It is possible that these point mutations may not have any functional consequence that contribute to autism since novel non-synonymous mutations have also been identified in 400 control individuals. Here in my work, I have explored the functional impact of some of these mutations in HEK293T cells and neurons.

Neurexin family of cell adhesion proteins

The human *NRXN* genes encode for neuronal cell adhesion proteins and includes three genes in the vertebrate family. Each gene encodes for a α-neurexin with a long extracellular domain and a β-neurexin with a shorter extracellular domain, which are generated from independent promoters (Ushkaryov *et al.* 1994). In sum, mammalian species have six principal forms of neurexins: α-neurexins (1-3) and β-neurexins (1-3) generated from two alternative promoters. Due to alternative splicing, the six individual promoters can generate thousands of neurexin isoforms, making neurexin one of the most polymorphic proteins in the human proteome. Diversity in alternative splice variants

suggest diversity in function. While the functional consequences of these isoforms are not well known, it is likely that various isoforms of neurexins can interact with different binding partners to fine tune neuronal functions. In contrast to mammalian neurexins, *Drosophila melanogaster* and *Caenorhabditis elegans* only have one α-neurexin, suggesting that β-neurexin arose later in evolution.

Neurexins are conserved evolutionarily and are closely related between different species (Missler *et al.* 1998). Bovine and rat neurexins exhibit 99% homology in protein sequence, suggesting a strong evolutionary pressure for sequence conservation between species (Missler *et al.* 1998). Neurexins are only found in brain based on northern blotting analysis, suggesting that they are neuronal specific proteins (Ushkaryov *et al.* 1992). Since there are many different isoforms due to alternative splicing, it raises the question whether these isoforms have brain specific distribution and function. Based on *in situ* hybridization analysis, neurexins have differential expression in brains with some degree of overlap, suggesting that they have differential regulation (Ullrich *et al.* 1995). However, α-neurexin and β-neurexin from the same neurexin gene are very different in their distribution, suggesting independent regulation and functional specification. In general, most neurons express multiple isoforms of neurexins (Ullrich *et al.* 1995).

Structure of neurexins

Neurexins are type I single pass transmembrane proteins with a modular architecture, suggesting that they were sequentially assembled during evolution. All neurexins have the same basic domain structure. Structurally, α-neurexin contains a

cleavable signal peptide followed by three cassette repeats, a transmembrane domain, and an intracellular tail (Missler *et al.* 1998)—see Fig. 1.6. α-Neurexin has six laminin-neurexin-sex hormone (LNS) binding globulin and three epidermal growth factor (EGF) domains (Missler *et al.* 1998). The three repeats of LNS(A)-EGF-LNS(B) cassette are the hallmark of α-neurexin. β-Neurexins contain only part of the last cassette, therefore resulting a much shorter extracellular domain. The cytoplasmic tails are identical for both isoforms. Neurexins have relatively short cytoplasmic tails with a PDZ-binding motif, which are important for interaction with other synaptic proteins CASK (Hata *et al.* 1996), syntenin (Grootjans *et al.* 2000), and Mints (Biederer and Südhof 2000). The PDZ-binding motif is also required for neurexin trafficking (Fairless *et al.* 2008). Deletion of the last four amino acids of PDZ-binding motif traps neurexins in the neuronal soma.

Function of neurexins

Neurexin Iα, the first neurexin identified, binds to α-latrotoxin, a potent excitatory neurotoxin from black widow spider to trigger vesicular release at the presynaptic terminal (Sugita *et al.* 1999). Other neurexin isoforms have later been identified because of their sequence homology to neurexin Iα (Ushkaryov *et al.* 1992). Neurexins also bind to neuroligins, dystroglycan, and neurexophilins at the cell membrane. These interactions may or may not require calcium. Neuroligin binding to neurexin (both α- and β-neurexin isoforms) requires extracellular calcium (Missler

2003). Dystroglycan binding to α-neurexin is more favorable with calcium. Neurexophilin binding to α-neurexin is calcium-independent.

Neurexins are involved in synapse formation; e.g., presynaptic β-neurexins binding to postsynaptic neuroligin-1 trigger synapse formation (Dean *et al.* 2003). Also, neuroligins-1 and -2 expressed in non-neuronal HEK293 cells induce presynaptic structure in contacting axons (Scheiffele *et al.* 2000). However, this finding is controversial since different experiments reveal contradicting results. One study found that triple knockdown of neurexins in cultured hippocampal mouse neurons did not alter synapse formation between neurons, but inhibited heterologous synapse formation (Gokce and Südhof 2013). Another study found that neurexin triple knockdown neurons could still induce proper synapse formation between neurons, suggesting that either neurexins are not essential for synapse formation or the knockdown was incomplete. Based on these results, whether neurexins are involved in synapse formation or not is up to debate. The contradiction can be explained by the differences in experimental setups since cultured neurons have more compensatory mechanisms compared to the simplified heterologous cell culture with non-neuronal cells. What is certain is that the mechanism of synapse formation may be more complex than what we think.

Neurexins are involved in synapse organization and differentiation. Independent of neuroligins, clustering of β-neurexins at the presynaptic terminal recruit cytoplasmic scaffold and assemble presynaptic terminals (Dean *et al.* 2003), suggesting that neurexins alone are sufficient to form hemisynapses. Also, β-neurexins expressed on non-neuronal cells can induce postsynaptic clustering of PSD95 in neurons, suggesting that β-neurexins

are involved in postsynaptic organization (Nam and Chen 2005). Moreover, β-neurexins induce postsynaptic differentiation of inhibitory GABAergic and excitatory glutamatergic synapse, suggesting that neurexins are involved in synapse specification (Graf *et al.* 2004).

Neurexins are involved in neurotransmission of both excitatory and inhibitory synapses (Zhang *et al.* 2005, Etherton *et al.* 2009, Zhang *et al.* 2010). α-Neurexins triple knockout mice have lower release frequency in both excitatory AMPA- and inhibitory $GABA_A$-receptor-mediated spontaneous neurotransmitter release (Missler *et al.* 2003). Whole-cell recording in neocortical slices from α-neurexins knockout mice revealed that a reduction in NMDA-receptor-mediated postsynaptic current (Kattenstroth *et al.* 2004). β-Neurexins are less essential for neurotransmission as compared to α-neurexins (Born *et al.* 2015), and this is probably because β-neurexins are less abundant in the brain (Schreiner *et al.* 2015).

Lastly, a more novel function of neurexin in signaling has surfaced recently. Neurexin I can bind to adhesion-type G-protein-coupled receptors (GPCRs) on the postsynaptic membrane (Boucard *et al.* 2012). However, the functional significance of this interaction is not well known. In summary, neurexins are involved in synapse formation, synapse differentiation, synaptic transmission and signaling.

α-Neurexins are essential for postnatal survival as triple knockout (KO) of all three α-neurexins dramatically reduces postnatal survival rate in mice. This lethality is caused by the loss of Ca^{2+} channel function in the absence of α-neurexins, which are essential for Ca^{2+} channel organization at the presynaptic release site. Even single α-

neurexin KO mice exhibit impaired survival rate, which suggests that α-neurexins have essential but redundant functions. Also, these mice exhibited altered synaptic transmission. However, synaptic morphology appeared to be normal. Another study found that mouse neurexin Iα deletion causes electrophysiological and behavioral changes consistent with some features of cognitive impairments (Etherton *et al.* 2009).

Mutations in neurexin links to autism

Altered synaptic function has emerged as a strong basis for impaired cognition and cause for ASD which is supported by the prevalence of highly penetrant human mutations in genes such as neurexin I and neuroligins, which are associated with synaptic structure and function (Feng *et al.* 2006, Kim *et al.* 2008, Zoghbi and Bear 2012, Südhof 2008). The human *NRNX1* gene, located on chromosome 2p16.3 is one of the largest genes in the genome. Due to the recent advancement in high throughput array-based genome screening technologies, many rare copy number variants have been identified in large cohort population (Diaz de Stahl *et al.* 2008). Notably, copy number variants in *NRXN1*, *NLGN3,* and *NLGN4* that are involved in synapse structure and function have been identified (Kumar and Christian 2009).

Copy number variants (CNVs) in the human *NRXN1* gene have been genetically linked to autism and schizophrenia (Rujescu *et al.* 2009). Large *de novo* copy number variant contributes to 10% of autism spectrum disorders (Sebat *et al.* 2007, Zhao *et al.* 2007, Weiss *et al.* 2008). In addition, other chromosomal abnormalities such as

rearrangement in *NRXN1*, *de novo* heterozygous deletion of *NRXNI,* and rare sequence variants in β-*NRXN1* have been associated with autism (Kim *et al.* 2008).

Thesis rationale

ASDs are heterogeneous neurodevelopmental disorders with a broad pathological or neurobiological etiology. Chromosome 15q11-q13 has been identified as a strong candidate region for autism susceptibility based on the frequent occurrence of chromosomal abnormalities and numerous linkage and genome-wide association studies (Mann *et al.* 2004, Wang *et al.* 2004). *APBA2*, also known as *MINT2*, is located at the distal region of 15q11-q13, which spans several hundred thousand base pairs and consists of a handful of neuronal genes. *MINT2* encodes for a neuronal adaptor protein that is involved in various important synaptic function. Mint2 is implicated as an important mediator of ASDs and neuropsychiatric phenotypes based on several lines of evidence. First, the *MINT2* gene maps to the distal portion of chromosome 15q13.1, a region commonly deleted in Prader-Willi and Angelman syndromes (neurodevelopmental disorders) and duplicated in cases of autism (Babatz *et al.* 2009), suggesting that the appropriate gene dosage of *MINT2* is important for neuronal development and brain function. Second, several studies have independently identified copy number variants (CNVs) in the *MINT2* gene in autism (Iossifov *et al.* 2014, De Rubeis *et al.* 2014). Third, seven nonsynonymous coding variants have been identified in the *MINT2* gene associated with autism (Babatz *et al.* 2009). Lastly, Mint2 interacts directly with neurexin I (*NRXN1)*, an ASD gene, as part of a multi-protein complex that acts as a facilitator of

neurotransmitter release at the synapse (Südhof 2008). Neurexin Iα deletion leads to electrophysiological and behavioral changes that contribute to cognitive impairments that are associated with autism (Etherton *et al.* 2009). Interestingly, Mint2 binds to neurexin I to stabilize synapse assembly (Biederer and Südhof 2000). Together, these data strongly suggest that copy number and sequence variations in Mint2 may contribute to neuronal dysfunction in autism. Despite the central importance of neurexin, little is known about the functional role of Mint2-neurexin interaction and its association to autism. Therefore, I hypothesized that Mint2-neurexin plays an important role in neuronal development and function and alterations of this interaction may contribute to ASD pathogenesis.

For this study, the effects of Mint2 copy number and Mint2 ASD sequence variant N723S in neuronal development and function were examined (Fig. 1.7). Copy number variants are the duplication or deletion of DNA, which leads to more or less genetic material, respectively. Both types of Mint2 copy number variants were studied. Mint2 copy number variant (duplication of DNA) was mimicked by Mint2 overexpression in wild type neurons. Mint2 copy number variant (deletion of DNA) was mimicked by Mint2 knockout in Mint-deficient neurons. Mint2 ASD sequence variant N723S was also studied in Mint-deficient neurons. The study found that Mint2 copy number variants (duplication and deletion of Mint2) and Mint2 N723S sequence variant lead to deficits in neuronal development and synaptic function.

Figure 1.1: Candidate genes for autism spectrum disorder (ASD) and intellectual disability (ID) are converged into three common molecular pathways, chromatin remodeling, Wnt signaling, and synaptic function.

In the synaptic function pathway, Mint1 (*APBA1*), neurexin I (*NRNX I*), and neuroligin (*NLGN I*) among others were pulled out from the screen. *De* novo truncation mutations are in red nodes. Missense mutations are in blue nodes. Number of cases is indicated by the size of the node. *Figure from Krumm, 2013.*

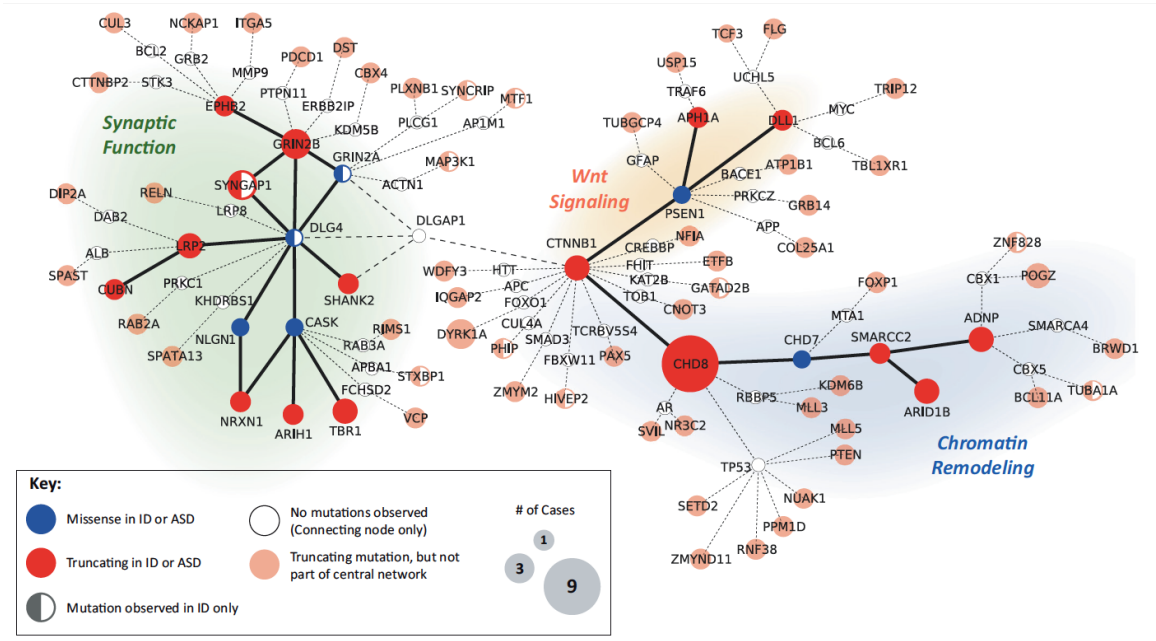


Figure 1.2: Autism candidate genes are co-expressed in modules.

Correlation module showing genes, such as *APBA2*, *CNTNAP1*, *CAMK2B* and many others are co-regulated in autism. These genes are involved in synaptic function, vesicular transport, and neuronal projection, suggesting that a complex interplay of many genes from multiple pathways are involved in autism. *Figure from Voineagu, 2011.*

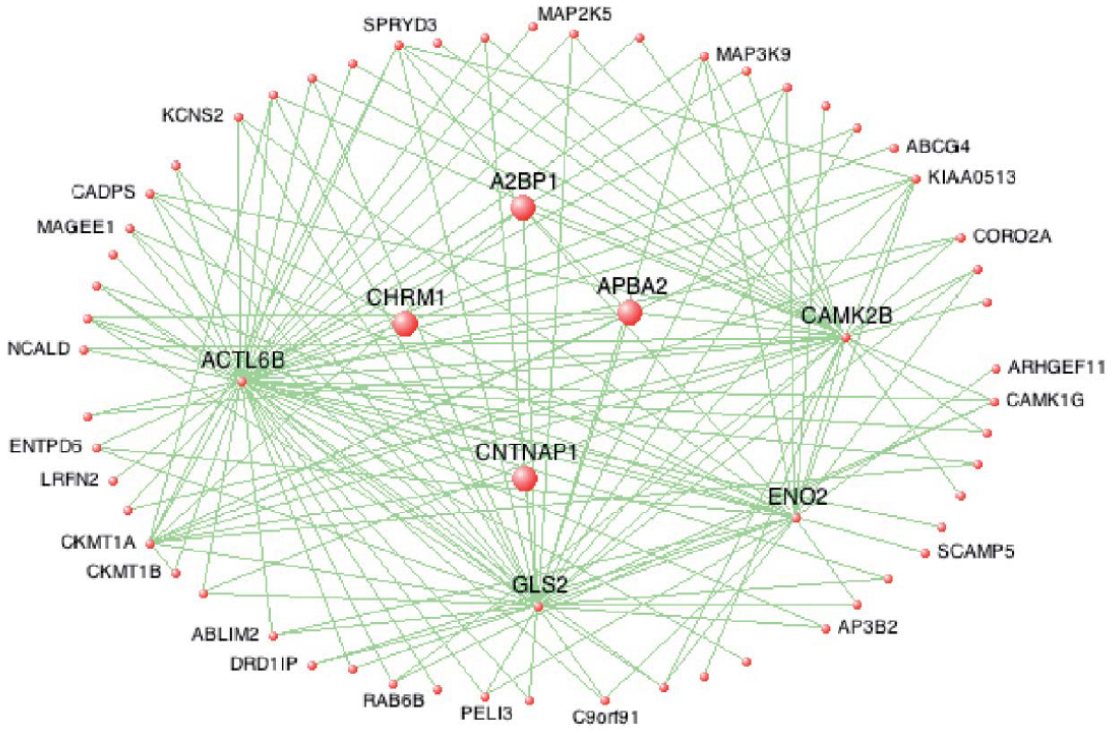


Figure 1.3: Structure of Mints.

The human Mint family of adaptor proteins are multidomain proteins that consists of a divergent amino (N)-terminus and conserved carboxyl (C)-terminus. Mint1, the largest of all Mints, has the CASK-interacting domain (CID) in the N-terminus. Also in the N-terminus of Mints 1 and 2, the Munc-interacting domain (MID) binds to Munc18, an essential synaptic vesicle protein that links Mints to synaptic vesicle exocytosis. In the conserved C-terminus, the phosphotyrosine binding (PTB) domain of all three Mints bind to the cytoplasmic tail of the APP and alter its proteolytic processing which is centrally involved in the pathogenesis of Alzheimer's disease. All Mints have two postsynaptic density-95/*Drosophila* discs-large/zona occludens-1 (PDZ) domains that bind to various proteins *in vitro,* including neurexin I, presenilin I, and calcium channels. Amino acid residue numbers are indicated on the end of each Mint protein.

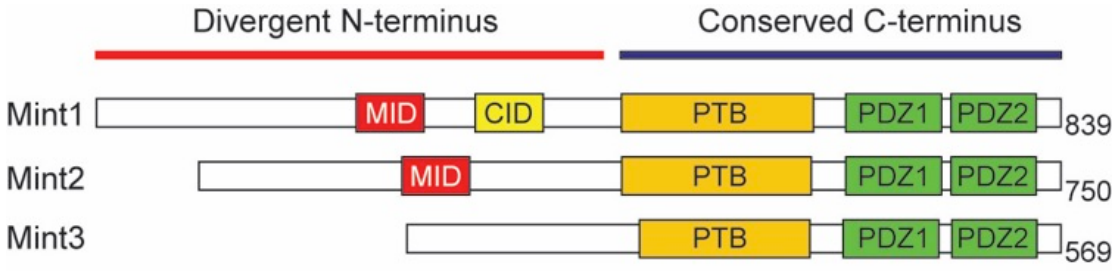


Figure 1.4: Diagram of possible interactions of Mints 1 and 2 with other synaptic proteins.

Schematic diagram showing that Mints 1 and 2 can form multi-molecular complexes with other synaptic proteins, such as CASK, Munc18 (M18), and neurexin on the presynaptic membrane. The diagram on the left showing that neurexin (embedded in the plasma membrane) binds the PDZ domains of Mint1, which also interacts with Munc18 and CASK. The diagram in the middle showing that neurexin binds to the PDZ domains of Mint2, which also interacts with Munc18. The diagram on the right showing that neurexin binds to the PDZ domain of CASK, which also interacts with Mint1 and Munc18. The diversity of these multi-molecular complexes suggests diversity in function. *Figure from Biederer, 2000.*

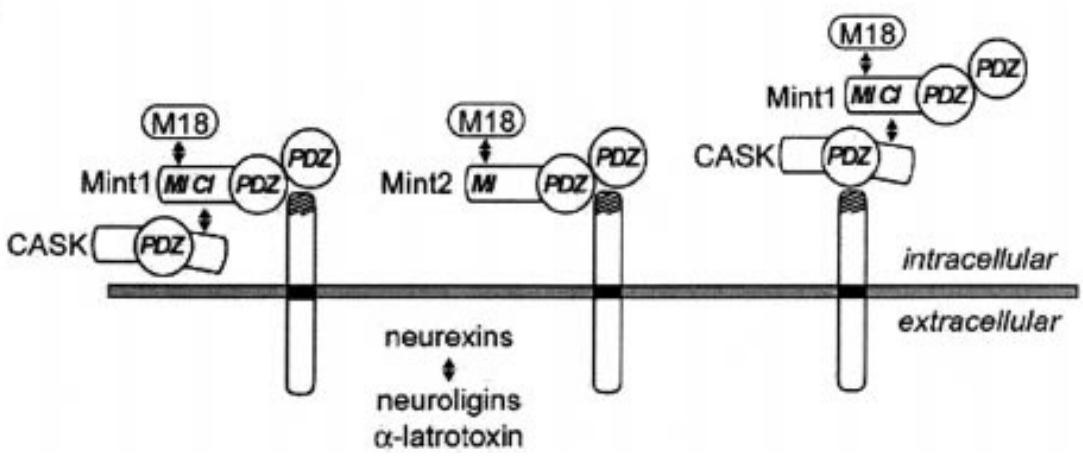


Figure 1.5: Diagram of chromosome 15q13 region and examples of *MINT2 (APBA2)* copy number variants found in autism, schizophrenia, and mental retardation.

An ideogram showing chromosome 15 and the genes (in blue) that are located in the 15q13 region. The *MINT2* gene (*APBA2*) is located in 15q13.1—highlight in yellow. The diagram showing different examples of copy number variants in chromosome 15. Copy number variants are the insertion or deletion of DNA fragments, typically from 50 base pairs up to several megabases. Insertion of DNA fragment would suggest more gene dosage and leads to protein overexpression. Deletion of DNA fragments would suggest less gene dosage and leads a partial knockout phenotype. The most common duplications are interstitial dup15q11.2q13.1 and idic(15). Other less common copy number variants, such as duplication (in green) and deletion (in red) are also found in individuals affected with autism, schizophrenia (SZ), and mental retardation (MR). *Figure from Babatz, 2009.*

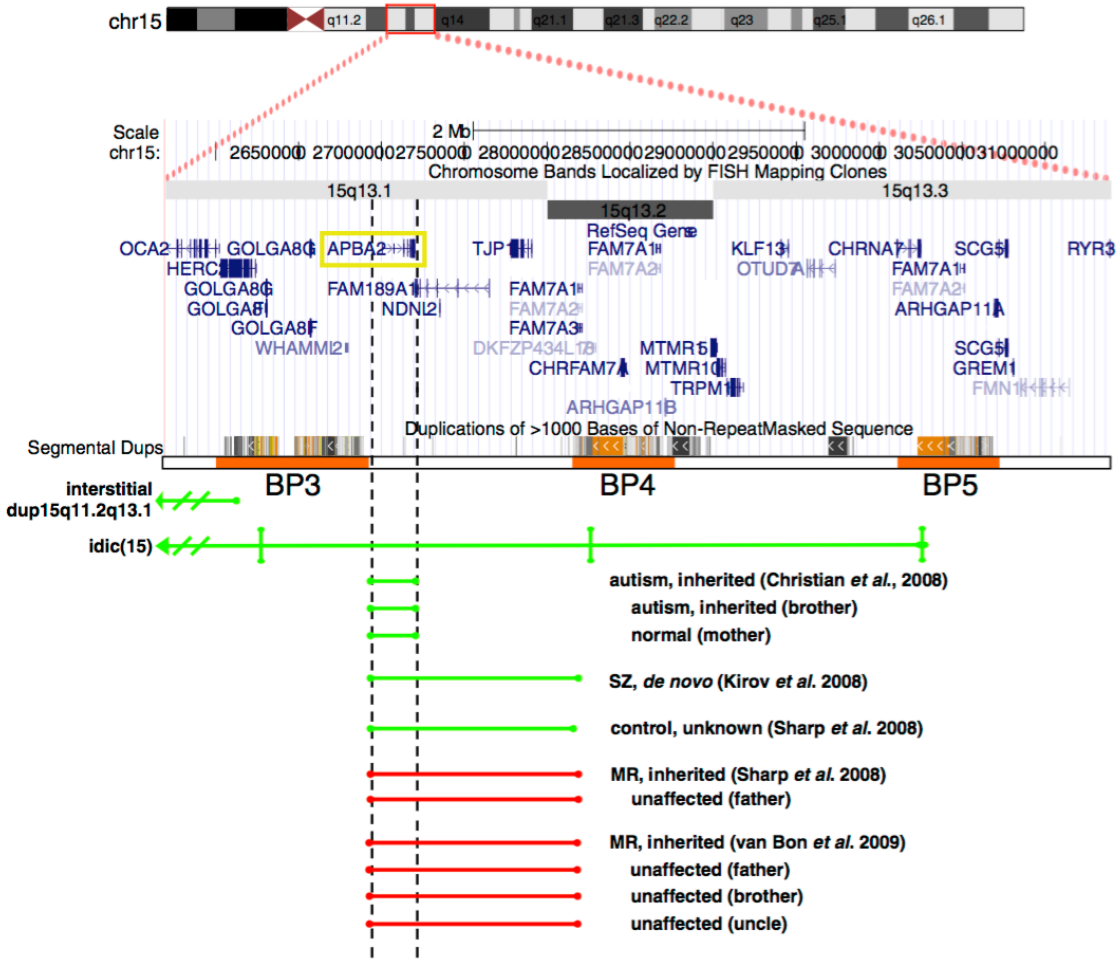


Figure 1.6: Schematic diagram of α-Neurexins and β-Neurexins structure.

α-neurexin starts with a cleavable signal peptide (SP) followed by three cassette repeats (marked I, II, and III), an O-linked carbohydrate modification (CH), a transmembrane domain (TMR), and an intracellular tail. α-Neurexin has six LNS (laminin-neurexin-sex hormone binding globulin) and three EGF (epidermal growth factor) domains. The three repeats of LNS(A)-EGF-LNS(B) cassette are the hallmark of α-neurexin. β-neurexins contain only part of the last cassette, therefore resulting a much shorter extracellular domain. Regions of alternative splicing sites are indicated with arrows. The cytoplasmic tails are identical for both isoforms. *Figure from Missler, 1998.*

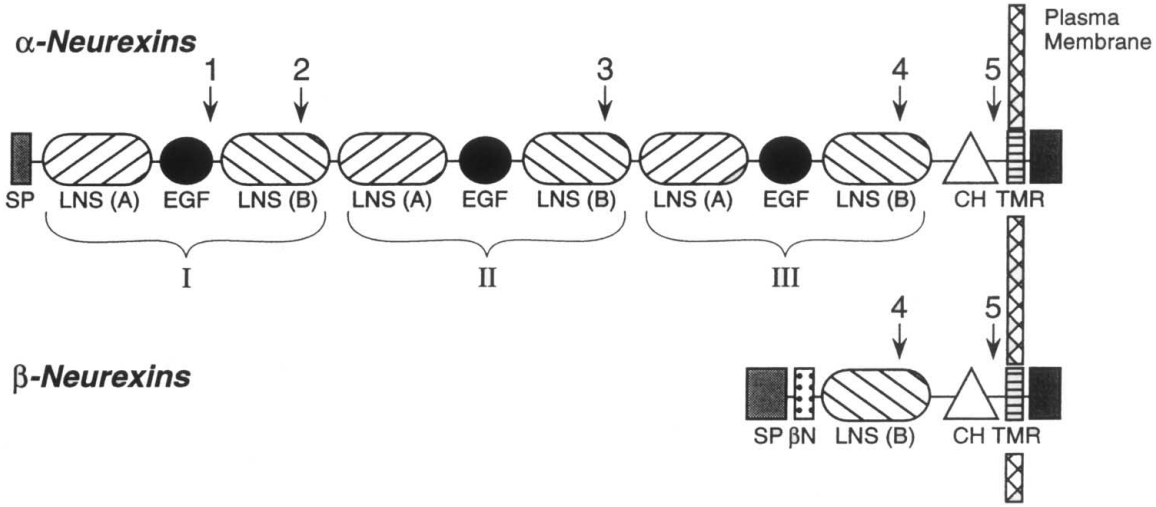
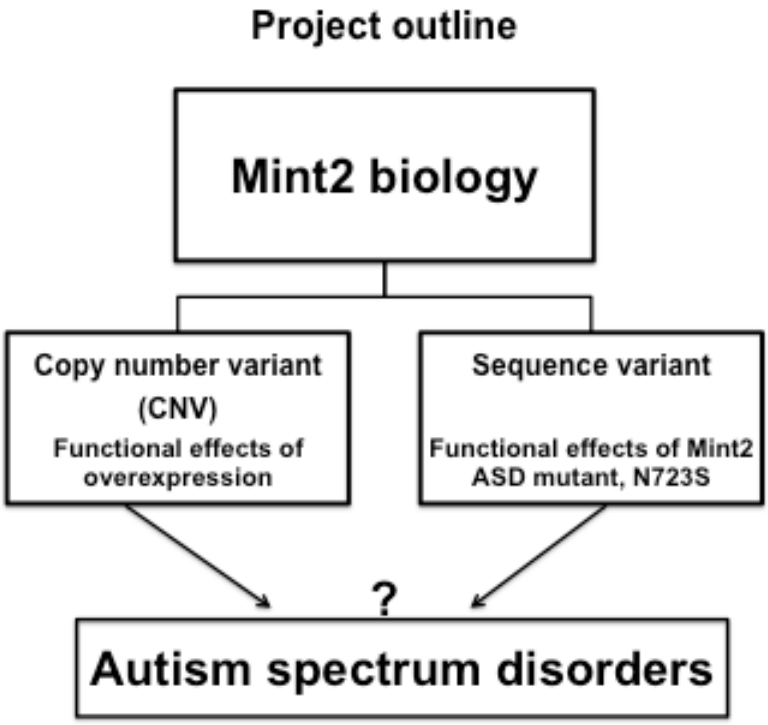


Figure 1.7: A schematic diagram of the projection outline to determine the effects of Mint2 copy number and sequence variant Mint2 N723S in neuronal development and function.

Mint2 copy number variant was emulated by the overexpression of Mint2 protein in wild type neurons. Mint2 N723S sequence variant was studied in Mint-deficient (in the absence of Mints 1-3) neurons with exogenous expression of GFP-tagged Mint2 N723S. Note that Mint-deficient neurons were used because Mints have redundant function.



CHAPTER TWO: Materials and Methods

Plasmids

All twelve nonsynonymous Mint2 mutations were made from pEGFP-C3-Mint2 by QuickChange Site Directed Mutagenesis (Agilent Technologies). The Mint2 PDZ1/2 truncation mutant (Mint2 ΔPDZ of 547 amino acids) was PCR amplified from pEGFP-C3-Mint2 and subcloned into pEGFP-C3. Afterwards, pEGFP-Mint2 wild type and mutants were digested with NheI and BamHI and subcloned into the lentiviral vector pFUW individually to make the recombinant lentiviral constructs. All plasmids were verified by sequencing. GST-neurexin I expression construct and pSyn5-E-mCherry-Neurexin Iα were kind gifts of Dr. Thomas Biederer (Tufts University, Boston, MA) and Dr. Markus Missler (Münster University, Germany), respectively. I also thank Dr. Anthony Boucard (Avandia Instituto Politecnico Nacinoa, Mexico**)** for the following plasmids: pCMV5-FLAG-neurexin Iα, pCMV5-FLAG-neurexin Iβ, pN1-mVenus, and pCMV5-neuroligin IΔAB-mVenus.

Primary neuronal cultures and lentiviral infections

Primary neuronal cultures were prepared from C57B6 (Jackson Laboratory) or CD1 (Charles River Laboratories) wild type or *M1*$^{-/-}$*; fM2/fM2; M3*$^{-/-}$ (*MINT1* and *MINT3* double knockout, *MINT2* conditional floxed knockout) newborn mouse pups of either sex. Dissociated brain tissues were trypsinized for 10 minutes (min) at 37°C and plated onto matrigel-coated (BD Bioscience, catalog # CB-40234) plates or coverslips (Ho *et al.*

2006, Ho *et al.* 2008). Cultured neurons were maintained in a humidified incubator with 5% CO_2 at 37°C. All animal experiments were performed in agreement with the Boston University Institutional Animal Care and Use Committee Guideline. Recombinant lentiviruses were generated in human embryonic kidney 293T (HEK293T) cells by cotransfection of pFUW-EGFP-ΔCre (a control vector without cre recombinase), pFUW-EGFP-Cre, pFUW-EGFP-Mint2 wild type, pFUW-EGFP-Mint2 N723S, or pFUW-EGFP-Mint2 ΔPDZ with viral enzymes and envelope proteins (pRSV/REV, pMDLg/RRE, and pCMV-VSVG) using FuGENE6 (Promega) (Ho *et al.* 2006, Ho *et al.* 2008). The initial media was changed into neuronal growth media after 8 hours (h) of transfection. Lentivirus-containing conditioned media was harvested after 48 h, centrifuged at 500 × *g* for 10 min at 4°C to remove cell debris, and stored at -80°C. Primary neuronal cultures from wild-type newborn pups were treated with either lentiviral control EGFP-ΔCre or EGFP-Mint2 wild type. Primary neuronal cultures from *M1*$^{-/-}$*; fM2/fM2; M3*$^{-/-}$ newborn pups were treated with either lentiviral control EGFP-ΔCre, EGFP-Cre alone, or EGFP-Cre together with EGFP-Mint2 wild type, EGFP-Mint2 N723S, or EGFP-Mint2 ΔPDZ.

Transient transfection of cell lines

HEK293T or COS-7 cells plated on 18 mm glass coverslips (Electron Microscopy Science, catalog # 7222-01) or tissue culture plates were grown to 70-80% confluency in 10% fetal bovine serum (Atlanta Biologicals) in DMEM (Invitrogen). Cells were transfected with Fugene6 reagent (Promega, catalog # PR-E2692). The DNA to Fugene6

ratio is 0.5 μg of DNA to 1.5 μl of Fugene6 for one well on a 12-well plate. 48 h post-transfections, cells on coverslips were fixed in 4% paraformaldehyde (Thermo Fisher Scientific, catalog # 28908) for immunocytochemistry. Cells on tissue culture plates were harvested with 2× reducing sample buffer with 10% β-mercaptoethanol for western blotting analysis.

Transient transfection of primary hippocampal neurons

Cultured hippocampal neurons at 7 or 9 days *in vitro* (DIV) were plated on 18 mm glass coverslips coated with matrigel and transfected with Lipofectamine 2000 transfection reagent (ThermoFisher Scientific, catalog # 11668027). DNA (1 μg) was added to 100 μl of Neurobasal media. In another tube, 6 μl of Lipofectamine 2000 transfection reagent was added to 100 μl of Neurobasal media. Both mixes were incubated at room temperature for 5 min. Then, the Lipofectamine transfection mix was added to the DNA mix and incubated for 20 min at room temperature. Neuronal conditioned media were saved and replaced with 500 μl of pre-warmed Neurobasal media. After 20 min of incubation, 200 μl of the DNA-Lipofectamine-neurobasal mix were added to neurons and incubated at 37°C for 30 min. After incubation, neurons were washed twice with 1 ml of pre-warmed Neurobasal media and replaced with the saved conditioned media. 48 hours post-transfection, neurons on coverslips were fixed with 4% paraformaldehyde for immunocytochemistry.

Co-immunoprecipitation

pCMV-APP was transfected together with GFP-tagged Mint2 wild type or Mint2 ASD mutations in HEK293T cells. 48 h after transfection, cells were washed with cold phosphate-buffered saline (PBS) and lysed. Lysates were solubilized for 30 min at 4°C and centrifuged for 10 min at max speed in a tabletop centrifuge. 10% of the supernatant were removed for Western blotting analysis as input. To the remaining 90% supernatant, rabbit anti-GFP antibody (1:500) was added and incubated overnight at 4°C. Protein A beads (Thermo Scientific, Catalog # 53142) were added for 2 h at 4°C. After binding, beads were washed thoroughly and 2× reducing sample buffer with 10% β-mercaptoethanol were added for Western blotting analysis.

GST pulldown

GST fusion neurexin I proteins were expressed in the BL21 (DE3) strain of *E. coli* and immobilized with glutathione sepharose beads (GE Healthcare, Catalog #17-0756-01) using a standard procedure. HEK293T cells were transfected with either GFP-Mint2 wild type or N723S. 48 h after transfection, lysates were solubilized in buffer containing 50 mM Tris-HCl, pH 7.2, 1 mM EDTA, 150 mM NaCl, 1% NP-40 and proteinase inhibitors. Lysates were rocked in 4°C for 30 min and centrifuged at 10 rpm at 4°C for 10 min. The supernatant was collected and incubated with 50% slurry of purified GST fusion neurexin I immobilized on glutathione sepharose beads for 2 h at 4°C. After incubation, beads were thoroughly washed three times with the same buffer as above and

2× reducing sample buffer with 10% β-mercaptoethanol was added. Samples were heat denatured and subjected to Western blotting analysis.

Native gel electrophoresis

HEK293T cells were first washed with PBS and solubilized in 300 μl/well of NativePAGE 4× sample buffer (Novex by Life Technologies, Catalog # BN2008) with a final concentration of 10% DDM (Novex by Life Technologies, Catalog # BN2005). After 15 min incubation on ice, cell lysates were homogenized by pipetting multiple times and centrifuged at 20,000 × g for 30 min at 4°C to separate the supernatant from the pellet. To prepare the loading sample, 5% G-250 sample additive was added to the supernatant so that the final G-250 concentration is 25% of detergent concentration. Native-PAGE Bis-tris gels electrophoresis was performed following the manufacture's protocol (Novex by Life technologies, Catalog # BN1002). Protein complexes were detected by Western blot.

Membrane and soluble neurexin fractionation

HEK293T cells transfected on a 10-cm dish were washed with 1× PBS and stored at -80°C (up to 3 months). Cells were warmed up in a 37°C water bath for 30 seconds (sec) to induce freeze-fracture of the cell membrane and homogenized with a syringe in 1.5 ml of 0.1% BSA in 1× PBS with proteinase inhibitors (aprotinin, pepstatin, and leupeptin). Fifty μl of this initial lysate was set aside for western blot analysis. The remaining lysate was spun down at 5,000 rpm for 5 min in a 4°C tabletop centrifuge to remove large

cellular debris. The supernatant was transferred to an ultracentrifuge tube (Beckman Coulter, catalog # 344057) and centrifuged at 135,000 × *g* for 1 h at 4°C. The supernatant "soluble" fraction was removed to a new tube and set aside for western blot analysis. The remaining pellet was solubilized in a buffer containing 1% Triton X-100, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 150 mM NaCl, 0.1% EDTA, and proteinase inhibitors and extracted for 2 h at 4°C. After extraction, the lysate was centrifuged at 100,000 × *g* for 1 h at 4°C. The supernatant "membrane" fraction was removed to a new tube for western blot analysis and the pellet "insoluble" fraction was discarded. Protein levels of all three fractions (total, soluble, and membrane) were quantified by immunoblotting analyses.

Immunocytochemistry and electron microscopy

Primary hippocampal neurons at 14 DIV were fixed with 4% paraformaldehyde at room temperature for 8 min, permeabilized and blocked in 10% goat serum and 0.1% saponin in PSB. Cells were incubated with primary antibodies in blocking buffer (10% goat serum in PBS) at 4°C overnight. Following PBS washes, cells were incubated with either goat anti-mouse or goat anti-rabbit IgG secondary antibodies conjugated to Alexa Fluor-546 or -647 (Invitrogen) in blocking buffer for 1 h at room temperature. Following PBS washes, coverslips were mounted with ProLong Gold Antifade Mountant with DAPI (Invitrogen) and imaged with a Carl Zeiss LSM700 confocal microscope. For electron microscopy, hippocampal neurons plated on coverslips at 14 DIV were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. Sample processing and image

acquisition were done by Dr. Xinran Liu (Yale University). Images were analyzed according to a standard protocol (Rosahl *et al.* 1995).

Electrophysiology

The spontaneous excitatory synaptic transmission was recorded from hippocampal neurons at 14–17 DIV via whole-cell voltage-clamping in the presence of 1 μM tetrodotoxin, 50 μM D-2-amino-5-phosphonovaleric acid, and 20 μM bicuculline. The pipette was filled with electrode solution containing 105 mM Cs-$MeSO_3$, 10 mM CsCl, 5 mM NaCl, 10 mM HEPES, 0.2 mM EGTA, 4 mM Mg-ATP, and 0.3 mM Na_2GTP, pH 7.4 (300 mOsm—adjusted with 1M sucrose). Recordings were obtained using an Axopatch 200A amplifier and 1440 Digitizer and analyzed with Clampfit software (Molecular Devices).

Heterologous synapse formation assay

Primary hippocampal neuronal cultures from *M1*$^{-/-}$*; fM2/fM2; M3*$^{-/-}$ newborn pups were treated with either lentiviral EGFP-Cre alone, EGFP-Cre with EGFP-Mint2 wild type, EGFP-Mint2 N723S, EGFP-Mint2 ΔPDZ or EGFP-Mint2 wild-type with EGFP-Mint2 N723S (heterozygous) at 3 DIV. COS-7 cells were transfected with mVenus control or NL1-mVenus using FuGene-6 (Roche) when neurons are at 10 DIV. 48 h after transfection, COS-7 cells were seeded onto the treated hippocampal neurons for 48 h of co-culturing to induce synapse formation at 12 DIV. After 2 days of co-culturing, cells were fixed with 4% paraformaldehyde and labeled with rabbit anti-synapsin P610

antibody and Alexa-546 secondary antibody. Z-stacked images were captured with a Carl Zeiss LSM700 confocal microscope and converted to the sum of slices using the ImageJ program (US National Institutes of Health). The degree of synapse formation between treatments was quantified as the ratio of presynaptic synapsin in neurons to postsynaptic mVenus in COS-7 cells. Briefly, an image of a NL1-mVenus transfected COS-7 cell was selected and the cell membrane (where most of the NL1-mVenus expression is localized) was traced in ImageJ program. The same trace was transferred to the synapsin image using the ROI Manager. The signal intensities for both images (synapsin and mVenus) were measured with the ImageJ and the ratio of signal intensity (synapsin/mVenus) was calculated for each treatment.

Fluorescent recovery after photo bleaching (FRAP)

Primary wild-type hippocampal neurons on glass bottom plate (*In Vitro* Scientific, Catalog # P06-20-1.5-N) infected with lentiviral Mint2 wild type or Mint2 N723S at 3 DIV were used for FRAP experiment at 14 DIV. A 488 nm diode laser was used to photobleach GFP-Mint2 enriched Golgi regions with 100% laser power for 3 consecutive cycles. Recovery fluorescence was acquired with 1% laser power and imaged every 5 sec for 170 sec. Analysis was generated by the FRAP module (Zen software, Zeiss).

Time-lapse imaging

Primary hippocampal neuronal cultures from *M1*$^{-/-}$*; fM2/fM2; M3*$^{-/-}$ newborn pups plated on glass bottom plates (*In Vitro* Scientific, Catalog # P06-20-1.5-N) were treated with

either lentiviral EGFP-Cre and EGPF-Mint2 wild type or EGPF-Mint2 N723S at 3 DIV. Images of neuronal processes expressing GFP-tagged Mint2 proteins were captured every 1 sec for 60 sec in a 37°C chamber with 5% CO_2 at 14 DIV. No visible signs of photodamage were observed because low laser power was used during imaging. Kymographs of straighten processes were created with ImageJ. The resulting kymograph shows distance travelled (on the x-axis) and time elapsed (on the y-axis).

Immunoblotting analysis

For immunoblotting analysis of neurons and cell lines, cells were washed with PBS and lysed with 2× reducing sample buffer with 10% β-mercaptoethanol. Harvested lysate was sonicated and boiled for 10 min. SDS-PAGE was performed using a standard technique. Proteins were transferred to 45 μm pore size nitrocellulose blotting membrane (GE Healthcare, catalog # 10600002) using a standard wet transfer system. The membrane was blocked with 5% milk in 1× Tris-buffered saline with Tween 20 (TBST) and probed with the appropriate primary antibody in the same blocking buffer overnight at 4°C. The membrane was thoroughly washed with 1× TBST and probed with either goat anti-rabbit or goat anti-mouse horseradish peroxidase (HRP) conjugated secondary antibody for 1 h at room temperature. After washing to remove the unbounded secondary antibodies, the membrane was incubated with ECL Prime Western Blotting Detection Reagent (GE Healthcare, catalog # 45-002-401) and XO-MAT film (Kodak) was exposed. Signal intensities were quantified with Image Studio Software (LiCor) and normalized for GAPDH or α-tubulin as a loading control on the same blot.

Statistical analysis

All error bars shown are mean ± standard error of the mean (SEM). Statistical significance between treatments was determined by pairwise Student's *t* test or One-Way ANOVA followed by Dunnett multiple comparison test (Prism 6, Graphpad).

Table 2.1: Plasmids

Description	Vector backbone	Method of cloning
Mint2 R4Q	pCMV3-EGFP	Site-directed mutagenesis
Mint2 G96_T98del-insA	pCMV3-EGFP	Site-directed mutagenesis
Mint2 A137V	pCMV3-EGFP	Site-directed mutagenesis
Mint2 H150D	pCMV3-EGFP	Site-directed mutagenesis
Mint2 Q436H	pCMV3-EGFP	Site-directed mutagenesis
Mint2 T660M	pCMV3-EGFP	Site-directed mutagenesis
Mint2 N723S	pCMV3-EGFP	Site-directed mutagenesis
Mint2 P22T	pCMV3-EGFP	Site-directed mutagenesis
Mint2 P105L	pCMV3-EGFP	Site-directed mutagenesis
Mint2 S114L	pCMV3-EGFP	Site-directed mutagenesis
Mint2 M476V	pCMV3-EGFP	Site-directed mutagenesis
Mint2 A632T	pCMV3-EGFP	Site-directed mutagenesis
Mint2 ΔPDZ	pCMV3-EGFP	Site-directed mutagenesis
Mint2 WT	pFUW	Restriction-based
Mint2 R4Q	pFUW	Restriction-based
Mint2 G96_T98del-insA	pFUW	Restriction-based
Mint2 A137V	pFUW	Restriction-based
Mint2 H150D	pFUW	Restriction-based
Mint2 Q436H	pFUW	Restriction-based
Mint2 T660M	pFUW	Restriction-based
Mint2 N723S	pFUW	Restriction-based
Mint2 P22T	pFUW	Restriction-based
Mint2 P105L	pFUW	Restriction-based
Mint2 S114L	pFUW	Restriction-based
Mint2 M476V	pFUW	Restriction-based
Mint2 A632T	pFUW	Restriction-based
Mint2 ΔPDZ	pFUW	Restriction-based

Table 2.2: Antibodies

Name	Source	Catalog #	Host	Dilution	Usage
Primary Antibodies					
Alpha 1 sodium potassium ATPase	Abcam	Ab7671	Mouse	1:200	WB
APP	Dr. Thomas Südhof	U955	Rabbit	1:1000	WB
APP 2C11	Millipore	MAB374	Mouse	1:1000	ICC
FLAG M2	Sigma	F3165	Mouse	1:1000	WB
GAPDH	Millipore	MAB374	Mouse	1:5000	WB
GFP	Dr. Thomas Südhof	437B	Rabbit	1:1000	WB
GFP	Synaptic System	132 002	Rabbit	1:1000	WB
GM130	BD Biosciences	610822	Mouse	1:1000	ICC
MAP2	Millipore/Chemicon	AB5622	Rabbit	1:1000	ICC
mCherry	Abcam	ab167453	Rabbit	1:500	ICC
Mint2	Sigma	M3319	Rabbit	1:1000	WB
Munc18	Dr. Thomas Südhof	K329	Rabbit	1:500	WB
Neurexin I	Synaptic Systems	174 103	Rabbit	1:1000	WB
PSD-95	Abcam	ab2723	Mouse	1:1000	ICC
SMI-312	Convance	SMI-312R	Mouse	1:1000	ICC
Synapsin	Dr. Thomas Südhof	P610	Rabbit	1:500	ICC
Synaptobrevin 2	Synaptic Systems	104 211	Mouse	1:1000	ICC
Tubulin (DM1A)	Cell Signaling	3873	Mouse	1:1000	ICC
Secondary Antibodies					
Anti-mouse Alexa 546	Invitrogen	A-11030	Goat	1:500	ICC
Anti-rabbit Alexa 546	Invitrogen	A-11010	Goat	1:500	ICC
Anti-mouse Alexa 647	Invitrogen	A-21236	Goat	1:500	ICC
Anti-rabbit Alexa 647	Invitrogen	A-21070	Goat	1:500	ICC
Anti-mouse HRP	Cell Signaling Technology	7076	Goat	1:5000	WB
Anti-rabbit HRP	Cell Signaling Technology	7074	Goat	1:5000	WB

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CHAPTER THREE: Characterization of autism-associated Mint2 mutations and their roles in neuronal development

Introduction

Autism is a neurodevelopmental disorder characterized by impairment in social interaction and language ability. *MINT2* is an autism candidate gene based on its strong genetic associations. The *MINT2* gene (also named *APBA2*) is located in the 15q11-q13 locus. Mutations in this locus have been frequently reported in autism (Cook *et al.* 1997, Babatz *et al.* 2009, Cook *et al.* 1998, Gillberg 1998, Schroer *et al.* 1998, Shao *et al.* 2002, Muhle *et al.* 2004, Christian *et al.* 2008).

The neuronal adaptor protein Mint2 (also named APBA2) is a multidomain protein with numerous binding partners. In the N-terminus, the Munc-interacting domain (MID) domain binds to Munc18-1 and facilitates vesicular fusion at the presynaptic terminal during neurotransmission (Okamoto and Südhof 1997). In C-terminus, the PTB domain binds to APP and modulates its metabolism associated with Alzheimer's disease (Tomita *et al.* 1999, Araki *et al.* 2003, Sano *et al.* 2006). The two PDZ domains in the C-terminus bind various proteins *in vitro*, including neurexin I (Biederer and Südhof 2000), presenilin I, and calcium channels (Maximov *et al.* 1999). These interactions strongly indicate Mint2 involvement in synapse function and neurodegeneration. In addition to *in vitro* studies, physiological functions of Mint2 have been examined in Mint2 knockout mice. Mints are involved in postnatal survival as 80% of Mint1/2 double knockout mice died at birth, but single knockout mice of either Mints survived normally, suggesting that

Mints are functionally redundant. Mint2 is also involved in normal emotional and social development (Sano *et al.* 2009). Mint2 knockout mice displayed conflict avoidance and subordination under competitive feeding situation. However, very little is known about Mint2 function in neurodevelopment and whether Mint2 contributes to autism.

Therefore, to determine whether Mint2 plays a role in neuronal development that may contribute to autism, I examined Mint2 function in both axonal and dendritic development. These analyses serve as a preliminary assessment to determine whether Mint2 is involved in neuronal growth. Based on histological studies of postmortem brains from autistic patients, neuronal overgrowth seems to be one of the contributing factors. In addition, I examined the effect of Mint2 copy number variant and specifically the ASD sequence variant N723S in axonal and dendritic development. The Mint2 copy number variant was examined by overexpression of Mint2 protein in wild-type neurons. The Mint2 ASD sequence variant N723S was studied in Mint-deficient neurons infected with GFP-Mint2 N723S lentivirus.

Results

Mint2 ASD mutants do not alter protein expression in HEK293T cells

A previous study has found seven nonsynonymous Mint2 mutations associated with ASD (Babatz *et al.* 2009) (Fig. 3.1A). Five of these seven coding variants in Mint2 were predicted to affect protein function (Babatz *et al.* 2009); however, these mutants have not been examined functionally. To determine whether Mint2 ASD mutants alter Mint2 protein expression, I generated a panel of lentiviral GFP-Mint2 mutants each

bearing one of the seven human Mint2 autism mutations or five control variants within the rat Mint2 cDNA since all the mutated residues are fully conserved between human and rat Mint2 (Fig. 3.1A). I evaluated whether Mint2 mutants were properly expressed *in vitro* by transfecting Mint2 wild type or mutants in HEK293T cells and immunoblotted for the GFP-tagged Mint2 protein (Fig. 3.1B). Since all the Mint2 plasmids are driven by the same CMV promoter, protein expression can be compared between wild type and mutations. Protein expression profile of GFP-Mint2 showed that the Mint2 mutants expressed at similar levels compared to Mint2 wild type suggesting that these Mint2 mutants did not disrupt Mint2 expression. GFP-tagged Mint2 was probed with anti-GFP antibodies. α-Tubulin was used as a loading control on the same blot.

Primary screening of Mint2 ASD mutants

Previously, Babatz and colleagues screened 512 autism probands and identified seven unique ASD Mint2 mutations and five control Mint2 mutations (Fig. 3.1A). Mint2 interacts with APP and regulates its processing. Several studies have shown a link between altered APP metabolism and abnormal intracellular accumulation of amyloid-β (Aβ) peptide in children and adults with autism (Frackowiak *et al.* 2013, Bailey *et al.* 2008, Sokol *et al.* 2006, Wegiel *et al.* 2012). In addition, APP is involved in neuronal development, neurogenesis, differentiation, and migration (Nicolas and Hassan 2014). First, to determine whether Mint2 ASD mutants alter APP protein expression, I co-transfected APP together with Mint2 mutants in HEK293T cells. I immunoblotted the harvested lysates for GFP-Mint2, APP, and GAPDH and found that Mint2 ASD mutants

did not alter APP protein expression (Fig. 3.2A). Next, to determine whether Mint2 ASD mutants alter APP binding, I have screened all Mint2 mutations in co-immunoprecipitation experiments with APP in HEK293T cells (raw data not shown, Fig. 3.2B-C showing three selective Mint2 ASD mutants). Based on the co-immunoprecipitation experiments with APP, I found that Mint2 R4Q, Mint2 T660M, and Mint2 N723S have the most dramatic and consistent changes compared to Mint2 wild type. The data revealed that while Mint2 T660M increases interaction with APP, Mint2 R4Q and Mint2 N723S decrease interaction with APP (Fig. 3.2B-C).

Since Mint2 interacts with neurexin I, I also screened all Mint2 mutations in GST pull down experiments with neurexin I (raw data not shown, Fig. 4.1A showing GFP-Mint2 N723S mutant). In addition, I have screened some of these mutants in a membrane fractionation assay to determine whether they alter neurexin membrane localization. The data revealed that Mint2 R4Q, Mint2 N723S, and Mint2 T660M all decreased the neurexin membrane localization compared to the wild type (Fig. 3.2D-E), suggesting that these mutations may be involved in neurexin membrane trafficking.

As a secondary screening in neurons, the effects of three mutants (Mint2 R4Q, Mint2 T660M, and Mint2 N723S) were also examined in both dendritic development with Sholl analysis (Fig. 3.3) and electrophysiological analysis (Fig 3.4). Both experiments indicated that Mint2 N723S mutant had the most pronounced phenotype compared to other mutants.

Based on these extensive experiments described above, Mint2 N723S mutation was selected for my study because of the following reasons: 1) Mint2 N723S is located

on the PDZ domain, which interacts directly with neurexin I (Biederer *et al.* 2002), a highly implicated protein in autism. However, the GST pull down assay indicates that Mint2 N723S does not alter neurexin interaction; 2) Mint2 N723S alters neurexin membrane localization in HEK293T cells, suggesting that Mint2 could be involved in neurexin trafficking and Mint2 N723S alters this function; 3) Mint2 N723S had the most dramatic phenotype in the Sholl analysis of dendritic growth compared to other mutants (Mint2 R4Q and Mint2 T660M); 4) Mint2 N723S had the most dramatic phenotype in electrophysiological analysis compared to other mutants (Mint2 R4Q and Mint2 T660M).

Mint2 N723S mutant alters axon length and dendritic branching

ASDs are associated with structural and functional abnormalities in neurons. To determine whether Mint2 N723S leads to impairments in neuronal development which are often associated with ASD phenotypes, I examined axonal and dendritic growth in primary neurons. Because Mints 1-3 have redundant functions and to examine the direct effects of Mint2 N723S mutant in neurons, I used a conditional knockout mouse line that is homozygous for Mint1 and 3 knockout and conditional floxed knockout for Mint2 (*Mint1*$^{-/-}$*; fMint2//fMint2; Mint3*$^{-/-}$*)*. Primary hippocampal neurons from *Mint1*$^{-/-}$*; fMint2//fMint2; Mint3*$^{-/-}$ newborn mice were cultured and I compared neurons that were co-infected at the day of plating with lentiviruses carrying cre and GFP-tagged Mint2 wild type, Mint2 N723S, Mint2 ΔPDZ or heterozygous Mint2 N723S mutation referred to as +/N723S. I wanted to determine whether Mint2 N723S alters the function of the PDZ domain, therefore, I used Mint2 ΔPDZ mutant was used as a negative control. As

analyzed by immunoblotting, I found that primary neurons infected with the various lentiviruses expressing GFP-tagged Mint2 variants showed similar protein expression levels compared to endogenous Mint2 levels (Fig. 3.5A).

To test whether the Mint2 N723S mutation alters axon polarization, I compared primary hippocampal neurons infected at 1 DIV with Mint2 wild type, Mint2 N723S, Mint2 ΔPDZ or +/N723S expressing lentivirus in Mint knockout neurons and immunolabeled at 3 DIV with SMI-312, a pan-axonal neurofilament marker. The quantitative analysis showed Mint knockout neurons decreased axon length compared with Mint2 wild type-infected neurons. By comparing Mint2 mutants with Mint2 wild type-infected neurons, neurons infected with Mint2 N723S, Mint2 ΔPDZ and +/N723S caused a decrease in the total axon length. Mint2 ΔPDZ mutant shown a similar decreased in axon length compared to the Mint2 KO, suggesting that the PDZ domains are involved in axon development. These results suggest that Mint2 has an important role in axon growth and the Mint2 N723S mutation leads to loss-of-function axonal impairments (Fig. 3.6A).

To determine whether Mint2 N723S leads to impairment of dendrite development, I infected *M1*$^{-/-}$*; fM2/fM2; M3*$^{-/-}$ hippocampal neurons at the day of plating with lentiviral cre recombinase alone, or cre recombinase with Mint2 wild type or N723S, and immunostained with a dendritic marker, MAP2, at 5 DIV. Sholl analysis was used to examine the complexity of the dendritic arbor and the overall pattern of arborization. The results revealed that Mint knockout neurons and the N723S mutant shifted the distribution to the left compared to Mint2 wild type infected neurons (Fig.

3.7A), indicating that Mint2 is involved in dendrite development and N723S mutant decreases dendritic branching.

Mint2 CNV promotes axon growth and dendritic branching

To determine whether Mint2 CNV leads to impairments in neuronal development, I characterized axon length and dendritic branching in wild type neurons in relation to different levels of Mint2 expression. Hippocampal neurons were infected with control (ΔCre) or Mint2 wild type lentivirus at the day of plating and immunostained with antibodies against an axonal marker (SMI-312) at 3 DIV or a dendritic marker (MAP2) at 5 DIV. The neuronal culture was infected with an increasing dosage of Mint2 wild type lentivirus, which correlates with increasing Mint2 protein levels on the immunoblot (ranging from 0 to 3 fold of protein level) (Fig. 3.5B). I found that Mint2 overexpression increased axon length compared to control (Fig. 3.6B). In addition, Sholl analysis revealed that Mint2 overexpression shifted the distribution to the right compared to Mint2 wild type infected neurons, indicating that Mint2 overexpression increased the total number of MAP2 positive processes (Fig. 3.7B). Altogether, these results suggest that overexpression of Mint2 is involved in both axon and dendrite development and Mint2 copy number variant promotes increased neuronal growth and is phenotypically similar to some patients with ASD.

Discussion

In the present study, I have showed the Mint2 ASD variants do not change Mint2 expression (Fig. 1C). This is not surprising, as most single-nucleotide polymorphisms (SNPs) do not change protein expression unless the point mutation encodes for a stop codon that leads to a premature stop in translation or for a key residue involved in protein folding. However, functional deficit may still occur without expression change. Since Mint2 is a multidomain adaptor protein, a mutation within a domain may interfere with Mint interacting with other proteins and therefore alter its function.

Based on the published study, it is not known whether Mint2 N723S is transmitted paternally or maternally because the parental information is not available in the database. Based on the NCBI SNP database, Mint2 N723S is not a common SNP. Therefore, I concluded that since Mint2 N723S is a rare point mutation in the study and in the general population, the individual is most likely to be heterozygous for the mutation. Therefore, to mimic the physiological condition of the patient, I also examined the effect of Mint2 N723S heterozygosity on neuronal development and function. However, the overarching goal of my project is to determine the function of Mint2 in neuronal development and autism, not necessarily the effect of one rare point mutation.

Mint2 is expressed earlier during gestation compared to Mint1 in mice (Ho *et al.* 2003), suggesting that Mint2 must have a temporal specific function in brain development. Mint2 copy number and sequence variants are associated with autism (Babatz *et al.* 2009), but its involvement in neurodevelopment is not known. In this

chapter, I examined whether Mint2 copy number and sequence variant N723S alter neurodevelopment.

Autistic individuals often have large brain size and too many synapses with a lack of synaptic pruning. Therefore, I first determined whether Mint2 copy number variant contributes to neuronal overgrowth. Copy number variant can either be gene deletion (leads to less protein production) or gene duplication (leads to more protein production). In this study, I only examined the effect of Mint2 copy number variant in term of gene duplication, by overexpression of Mint2 protein. I examined the role of Mint2 overexpression in axon growth and dendritic branching. The data revealed that Mint2 copy number variant increases axon length (Fig. 3.6B) and dendritic branching (Fig. 3.7B), suggesting that Mint2 overexpression promotes overall neuronal growth that can contribute to the physical changes of the brain that are associated with autism. More dendritic growth indicates more synapse formation during development, which correlates with the increase in frequency and amplitude in spontaneous excitatory neurotransmission (Sullivan *et al.* 2014).

Mint2 is well studied in the literature for its presynaptic function (Biederer *et al.* 2002, Ho *et al.* 2008). The Sholl analysis of dendritic growth suggests that Mint2 overexpression also has a postsynaptic function. However, a Mint2 dendritic function is not completely novel. Another study has found that Mint2 is involved in the dynamic of spine remodeling and Mint2 overexpression increases spine density (Jones *et al.* 2014). The increased in spine density suggests more synapse formation, which correlates well with the increases in dendritic branching I found in the Sholl analysis (Fig. 3.7B).

I also examined the effects of Mint2 N723S on axon and dendrite development. The data revealed that Mint2 N723S decreases axon length (Fig. 3.6A) and dendritic branching (Fig. 3.7A) similar to the Mint2 knockout, suggesting that Mint2 N723S decreases overall neuronal growth and alters neuronal development. To determine the precise domains that are involved, the PDZ domains deletion mutant was used as a control. The rationale was that if domains other than the PDZ were involved, there would be little or no change in axon development compared to the Mint2 knockout neurons. If the PDZ domains were involved in axon development, there would be a similar reduction in axon length as compared to the Mint2 knockout neurons. The result revealed that Mint2 ΔPDZ mutant reduces the axon length similar to the Mint2 knockout neurons (Fig. 3.6A). These results suggest the following: 1) Mint2 is involved in early neurodevelopment; 2) Mint2 N723S decreases axon development; and 3) PDZ domains are involved in the development of axon; 4) Mint2 N723S most likely alters the function of the PDZ domains; 5) It is interesting to note that Mint2 copy number variant (gain-of-function mutation) and Mint2 sequence variant N723S (loss-of-function mutation) have opposite phenotypes in axonal and dendritic development.

In summary, the effects of Mint2 overexpression and Mint2 knockout in axonal and dendrite growth clear indicate that Mint2 is involved in neuronal development, which is a novel finding. This also indicated that Mint2 has both presynaptic (i.e. axonal) and postsynaptic effects (i.e. dendritic). This is not fully surprising. Previously, Mint2 has shown to display polarized localization in somatodendrites and axons, and it assumes different functions depending on its localization (Nakajima *et al.* 2001). I speculate that

Mint2 copy number variant as a gain-of-function mutation and Mint2 N723S as a loss-of-function mutation. As of now, the precise mechanism of Mint2 in neuronal development is not known; however, since Mint2 is a neuronal adaptor protein, it may very likely traffic other proteins that are involved in axonal and dendritic growth.

Figure 3.1: Mint2 ASD mutants do not disrupt Mint2 expression in HEK293T cells.

A, The locations of nonsynonymous variants for Mint2 in ASD and control subjects. Human Mint2 mutants examined in this study include 7 unique ASD variants (above, red) and 4 control variants (below, black). The *A632T mutation represents one autistic proband and one control. *Figure adapted from Babatz, 2009*. ***B,*** Representative immunoblots of Mint2 protein levels in the cell lysate of HEK293T cells transfected individually with GFP-tagged Mint2 wild type or mutants.

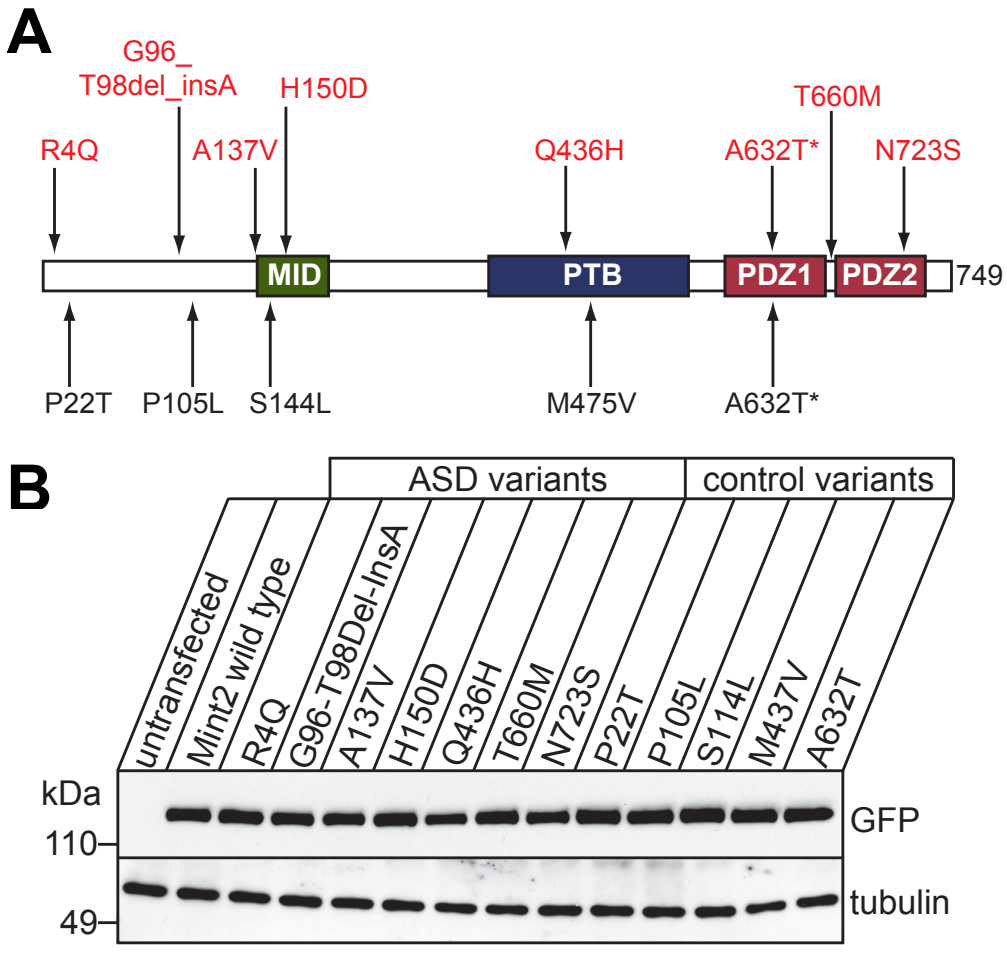


Figure 3.2: Characterization of Mint2 mutants in HEK293T cells.

A, Representative immunoblots of protein levels in the cell lysate of HEK293T cells. APP was transfected together with GFP-tagged Mint2 wild type or Mint2 mutants. Mint2 proteins were probed with rabbit anti-GFP antibody. APP proteins were probed with rabbit anti-APP antibody. GAPDH was used as a loading control on the same blot. ***B-C,*** Co-immunoprecipation experiment demonstrating that Mint2 mutants alter binding of Mint2 to APP in transfected HEK293T cells. APP was transfected together with GFP-tagged Mint2 wild type, Mint2 R4Q, Mint2 T660M, or Mint2 N723S mutant in HEK293T cells. Cell lysates were immunoprecipated with a rabbit anti-GFP antibody and immunoblotted with a rabbit anti-APP antibody. ***D-E*****,** Membrane and cytosolic fractions of cell lysates from HEK293T cells transfected with neurexin Iα and GFP-tagged Mint2 wild type, Mint2 R4Q, Mint2 T660M, or Mint2 N723S mutant.

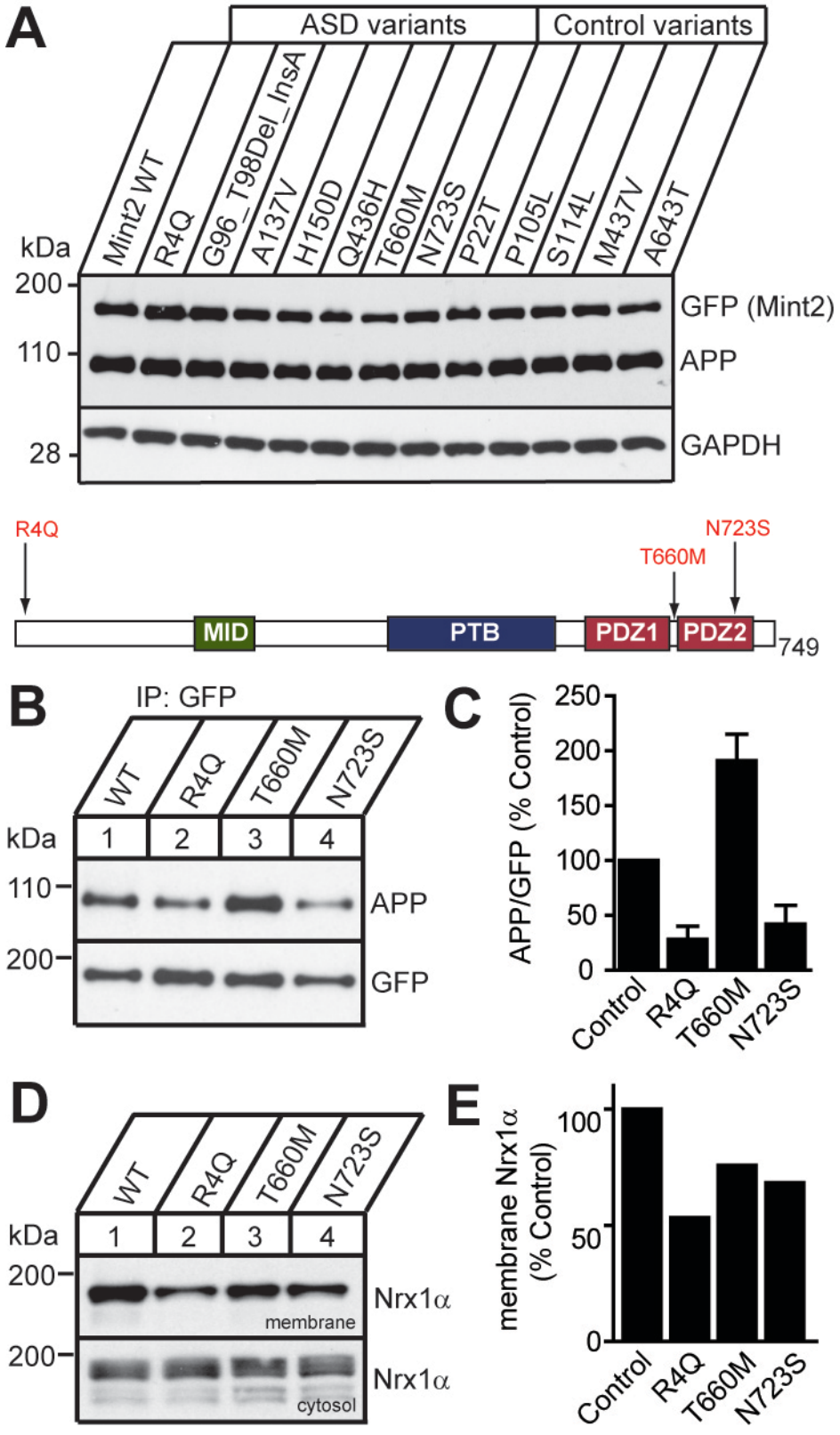

A
ASD variants
Control variants
Mint2 WT
R4Q
G96_T98Del_InsA
A137V
H150D
Q436H
T660M
N723S
P22T
P105L
S114L
M437V
A643T
kDa
200
110
28
GFP (Mint2)
APP
GAPDH
R4Q
T660M
N723S
MID
PTB
PDZ1
PDZ2
749
B
IP: GFP
WT
R4Q
T660M
N723S
kDa
1
2
3
4
110
200
APP
GFP
C
APP/GFP (% Control)
250
200
150
100
50
0
Control
R4Q
T660M
N723S
D
WT
R4Q
T660M
N723S
kDa
1
2
3
4
200
200
Nrx1α
membrane
Nrx1α
cytosol
E
membrane Nrx1α (% Control)
100
50
0
Control
R4Q
T660M
N723S

Figure 3.3: Characterization of selective ASD Mint2 mutants in dendritic development. Mint2 N723S mutant decreases dendritic branching compared to Mint2 wild type.

A-D, Mint2 N723S mutation decreases dendritic branching. Primary hippocampal neurons were infected with lentiviral cre recombinase alone (KO), lentiviral cre recombinase with Mint2 wild type (WT), Mint2 T660M, or Mint2 N723S mutant at the day of plating. Cells were stained with MAP2 (a dendritic marker) at 5 DIV. Sholl analysis based on the number of dendritic crossings distributed over the distance from the cell body in pixels (n=25-30 neurons for each group). * $p<0.05$. Scale bars, 20 μm.

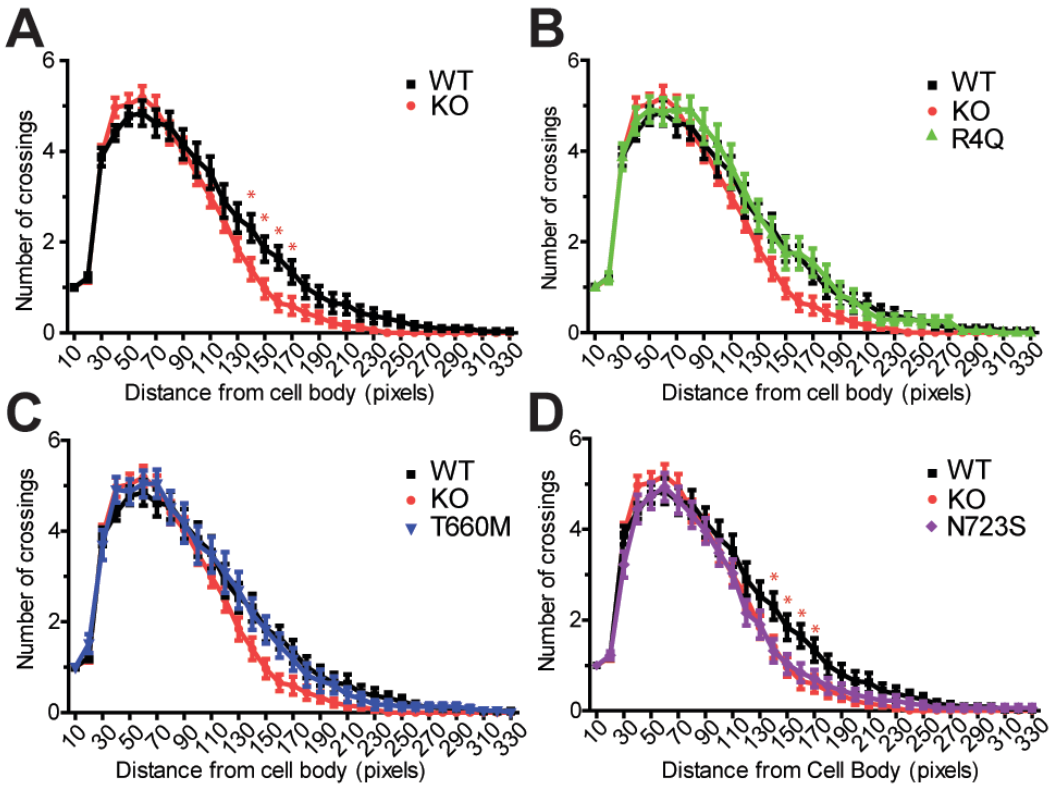


Figure 3.4: Characterization of selective Mint2 ASD mutants in neurotransmission. Mint2 N723S mutant decreases excitatory spontaneous neurotransmitter release in wild type neurons.

Bar graphs showing that Mint2 N723S had the most dramatic decreased in the frequency of mEPSC compared to the control (Mint2 wild type) and other Mint2 mutants (Mint2 R4Q and Mint2 T660M). The amplitude of mEPSC was not changed between all treatments.

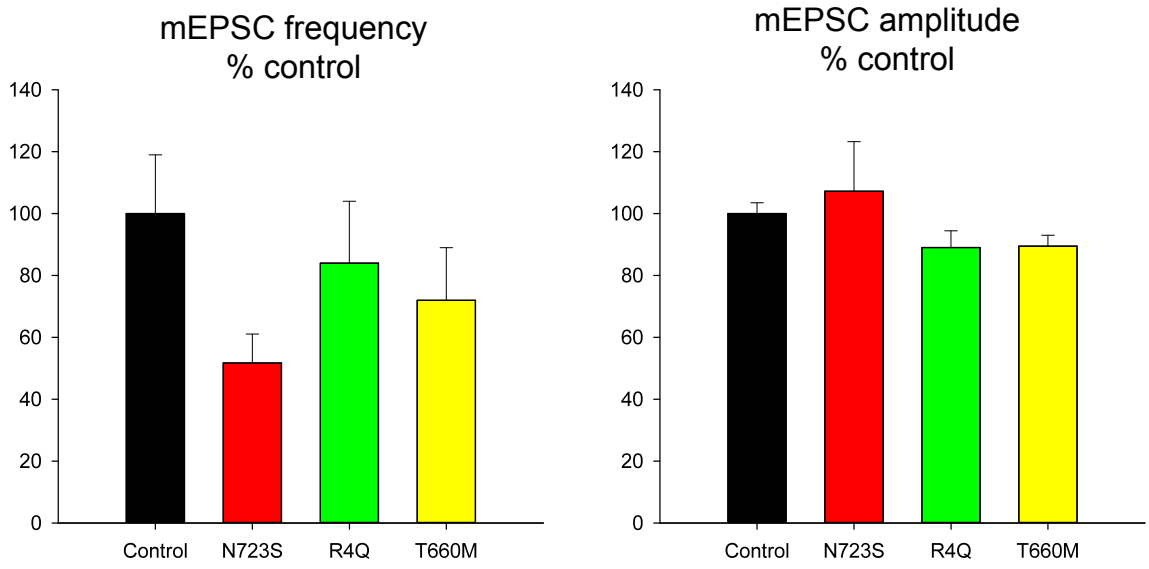


Figure 3.5: Protein expression levels of Mint2 wild type or mutations in lentivirus-infected neuron.

A, Representative immunoblot of Mint2 protein levels from cell lysates of *M1*$^{-/-}$*; fM2/fM2; M3*$^{-/-}$ neurons infected with Δcre (control for cre recombinase), cre recombinase alone, cre recombinase with and GFP-Mint2, N723S, ΔPDZ, or Mint2+/N723S (heterozygous mutant). To probe for the Mint2 protein, rabbit anti-Mint2 antibody was used here. ***B,*** Representative immunoblot of Mint2 protein levels from cell lysates of wild type neurons infected with increasing dosage of Mint2 wild type lentivirus, which correlates with increasing Mint2 protein levels on the immunoblot (ranging from 0 to 3 fold protein level increase). To probe for the Mint2 protein, rabbit anti-Mitn2 antibody was used here. GAPDH was used as a loading control on the same blot.

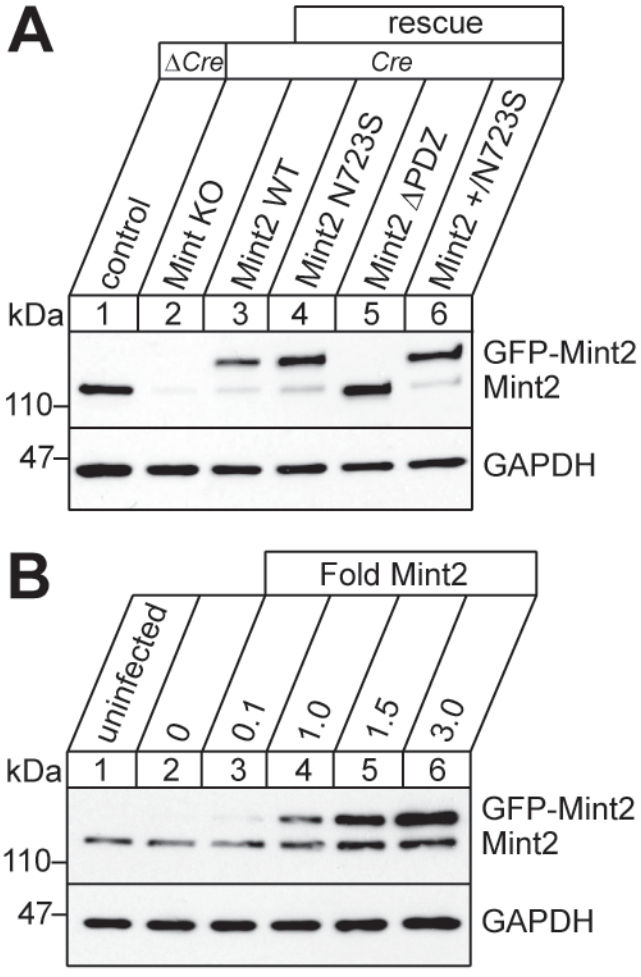


Figure 3.6: Mint2 sequence variant N723S and copy number variant alter axon development.

A, Mint2 N723S mutation decreases axon growth. Representative images of primary hippocampal neurons at 3 DIV infected with lentiviral cre recombinase together with with Mint2 wild type (WT), cre recombinase alone (KO), cre recombinase together with Mint2 N723S, Mint2 ΔPDZ, or heterozygous (Mint2+/N723S) mutant on the day of plating. Cells were stained with an axonal marker, SMI-312. Numbers in bars represent analyzed cells from 2 independent neuronal cultures (WT, n=34; KO, n=75; N723S, n=79; ΔPDZ, n=38; +/N723S, n=60). ***B,*** Mint2 CNV increases axon growth. Representative images of primary hippocampal neurons at 3 DIV infected with lentirivral control or Mint2 wild type and stained with SMI-312 (axonal marker) at 2 DIV. Numbers in bars represent analyzed cells from 3 independent neuronal cultures (control, n=85, Mint2 OE=75). Scale bars, 20 μm. *$p<0.05$; **$p<0.01$; ***$p<0.005$.

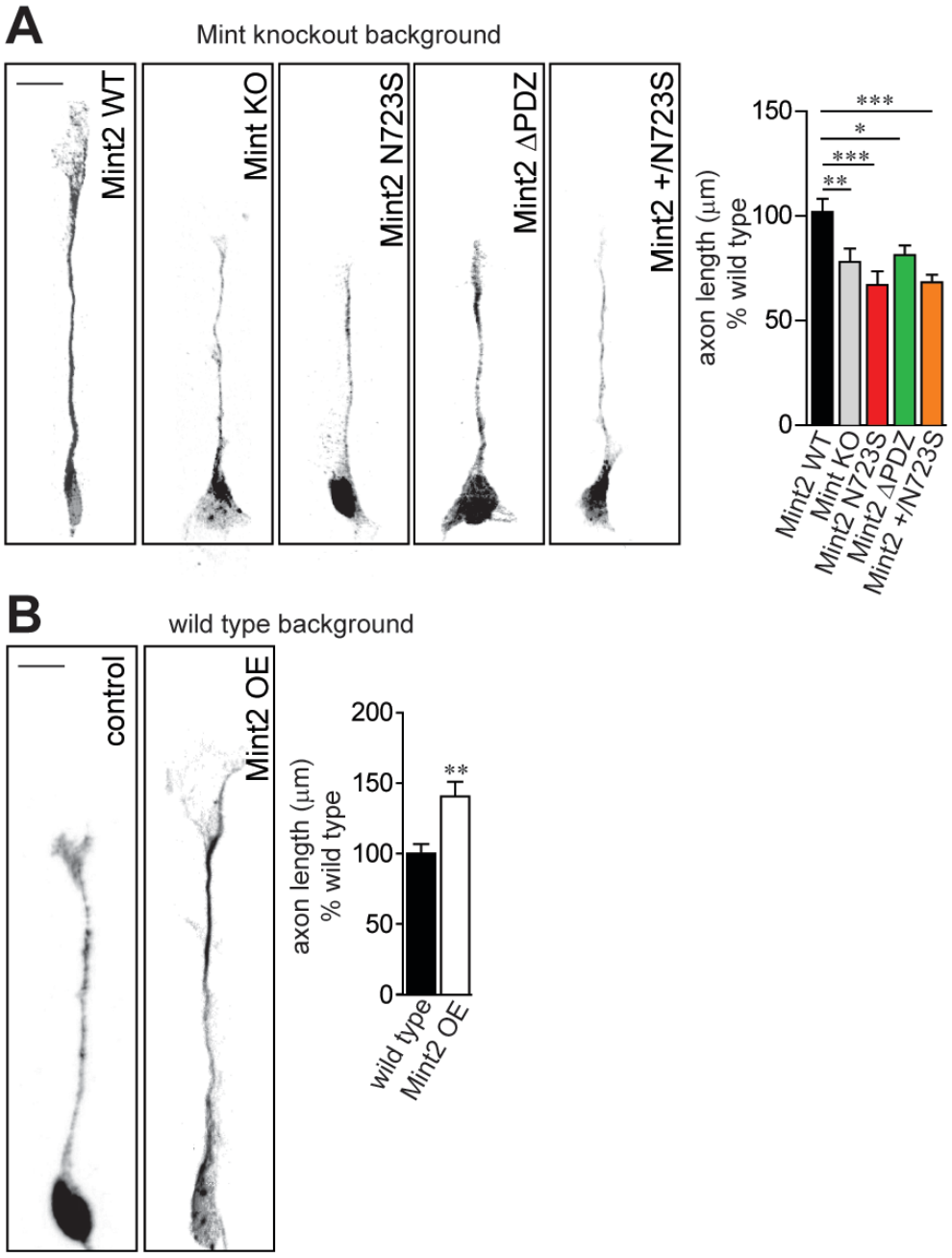
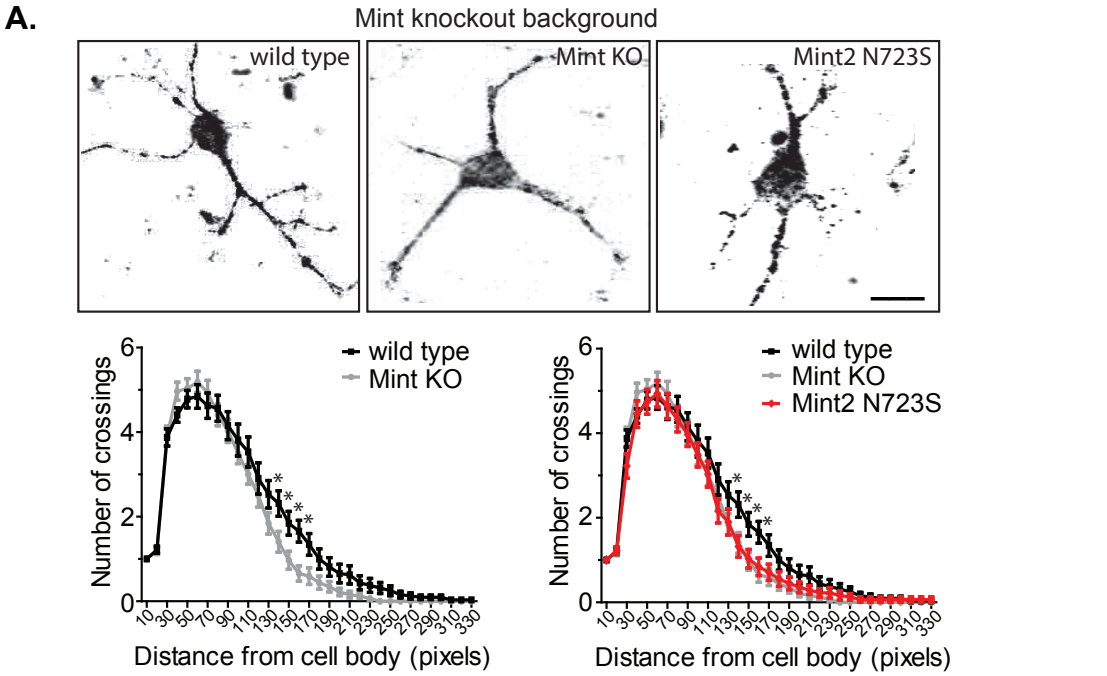
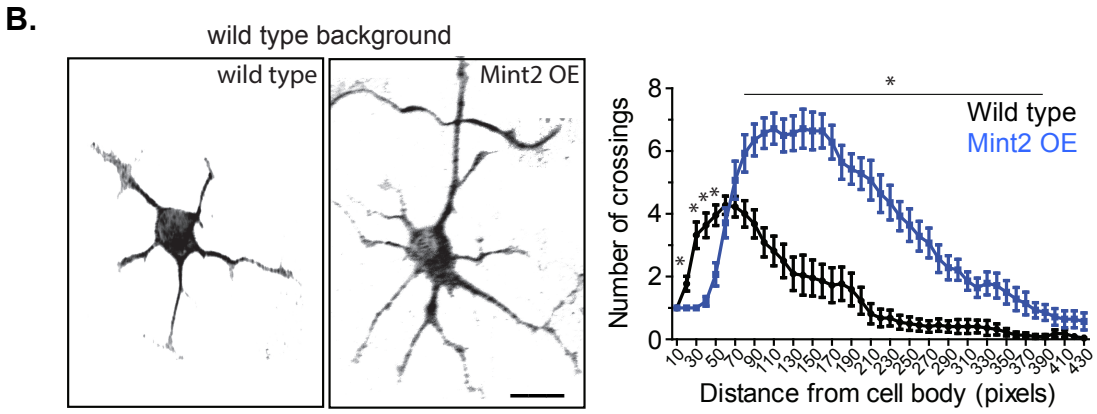

A
Mint knockout background
Mint2 WT
Mint KO
Mint2 N723S
Mint2 ΔPDZ
Mint2 +/N723S
axon length (μm)
% wild type
150
100
50
0
***
*
***
**
Mint2 WT
Mint KO
Mint2 N723S
Mint2 ΔPDZ
Mint2 +/N723S
B
wild type background
control
Mint2 OE
axon length (μm)
% wild type
200
150
100
50
0
**
wild type
Mint2 OE

Figure 3.7: Mint2 sequence variant N723S and copy number variant alter dendrite development.

A, Mint2 N723S mutation decreases dendritic branching. Representative images of Mint-deficient hippocampal neurons (Mint knockout background) infected with lentiviral cre recombinase alone (KO), cre recombinase together with Mint2 wild type or Mint2 N723S mutant on the day of plating. Cells were stained with MAP2 (a dendritic marker) at 2-3 DIV. Sholl analysis showed the number of dendritic crossings distributed over the distance from the cell body in pixels (n = 30 neurons for each group). ***B,*** Mint2 CNV increases dendritic growth and branching. Representative images of wild type hippocampal neurons (wild type background) infected with lentiviral control (wild type) or Mint2 wild type (Mint2 OE) on the day of plating. Cells were stained with MAP2 at 3-5 DIV. Sholl analysis showed the number of dendritic crossings distributed over the distance from the cell body in pixels (n = 20-30 neurons for each group). *$p<0.05$. Scale bars, 20 μm.


A.
Mint knockout background
wild type
Mint KO
Mint2 N723S
Number of crossings
wild type
Mint KO
Distance from cell body (pixels)
wild type
Mint KO
Mint2 N723S
Distance from cell body (pixels)


B.
wild type background
wild type
Mint2 OE
Number of crossings
Wild type
Mint2 OE
Distance from cell body (pixels)

.

CHAPTER FOUR: Mint2 N723S alters neurexin localization and trafficking

Introduction

Mint2 is a multidomain protein and its PDZ domains interact directly with neurexin I (Biederer, 2002), a neuronal cell adhesion protein that is highly implicated in autism. Neurexin is involved in numerous synaptic functions such as synapse formation and synaptic transmission. The proper membrane targeting of neurexin is important for its synaptic function. Neurexin membrane trafficking depends on its C-terminal PDZ-binding motif (Fairless *et al.* 2008), which interacts with PDZ-domain proteins, such as CASK and Mints. Previously, a study found that PDZ domains of Mint1 are involved in NMDA receptor subunit trafficking to the postsynaptic membrane (Setou *et al.* 2000). I hypothesize that Mint2 is involved in neurexin membrane trafficking, possibly via its PDZ domains. To test this hypothesis, I examined the role of Mint2 for neurexin membrane localization in HEK293T cells and synapse targeting in neurons.

Because the multi-domain structure and the nature of their interacting partners determine the function of Mint proteins, this raises the question whether Mint2 mutants alter the binding to any of its interacting partners and affect Mint2 function. I therefore analyzed the N723S mutation that targets a conserved asparagine residue in the second PDZ domain of Mint2 because the PDZ domains of Mint2 binds to Nrxn1α, a strong candidate gene for ASD.

Results

Mint2 N723S does not disrupt its binding to neurexin in HEK293T cells

Mints are multi-domain proteins and bind to several synaptic (Munc18-1, CASK, neurexin I, and Ca^{2+} channels) and Alzheimer's disease (AD)-related proteins (APP, presenilin 1) – see Fig. 3.1A**.** This raises the question of whether Mint2 mutants alter the binding to any of its interacting partners and affect Mint2 function. I therefore analyzed the effect of a single amino-acid substitution (N723S) that targets a conserved asparagine residue in the second PDZ domain of Mint2, because the PDZ domains of Mint2 bind directly to neurexin I, a strong candidate gene in autism (Südhof 2008). To determine whether the Mint2 N723S mutation alters its binding to neurexin I, I used the isolated cytoplasmic tail of neurexin I, immobilized as a bacterially expressed GST-fusion protein and tested whether neurexin I could pull down full-length Mint2 wild type, Mint2 N723S, or Mint2 ΔPDZ (PDZ domains deletion mutant) that were produced in transfected HEK293T cells. Consistent with previous studies, Mint2 wild type binds to Nrxn Iα whereas C-terminal truncation of the two PDZ domains of Mint2 (Mint2 ΔPDZ) dramatically reduced its binding to Nrxn Iα (Biederer and Südhof 2000). Meanwhile, the Mint2 N723S mutation did not disrupt Nrxn1α binding.

Mint2 N723S decreases neurexin membrane localization in HEK293T cells

Since Mints are involved in membrane protein trafficking to the cell surface (Chaufty *et al.* 2012, Matos *et al.* 2012, Sullivan *et al.* 2014), I examined whether Mint2

is involved in neurexin I trafficking and affects neurexin membrane localization. I co-transfected neurexin Iα together with GFP control, GFP-Mint2 wild type, GFP-Mint2 N723S, or GFP-Mint2 ΔPDZ in HEK293T cells. The proteins in the membrane and soluble fraction were separated by ultracentrifugation. In the membrane fraction, I found that Mint2 wild type significantly increases neurexin I compared to the GFP control, suggesting that Mint2 traffics neurexin to the membrane (Fig. 4.1B). In contrast, both Mint2 N723S and Mint2 ΔPDZ mutants decreased the membrane fraction of neurexin Iα compared to Mint2 wild type (Fig. 4.1B). Interestingly, I detected low cytosolic neurexin levels in Mint2 ΔPDZ mutant suggesting the ΔPDZ mutant may be sequestering neurexin to a non-functional, cytosolic compartment. Combined, these results indicate (1) Mint2 binds and traffics neurexin to the membrane, (2) Mint2 N723S mutant binds neurexin effectively, but it can't traffic neurexin to the membrane efficiently, and (3) Mint2 ΔPDZ mutant binds and traffics neurexin less efficiently.

To further validate Mint2-mediated neurexin Iα membrane localization, I visualized and quantified the membrane neurexin level by immunocytochemistry. I co-transfected mCherry-neurexin Iα together with GFP control, GFP-Mint2 wild type, GFP-Mint2 N723S, or GFP-Mint2 ΔPDZ in HEK293T cells. 24 hours post-transfection, cells were fixed without being permeabilized to allow the visualization of plasma membrane neurexins only. Cells were labeled with rabbit anti-mCherry antibody to amplify the mCherry signal. Similar to the result I found in cellular fractionation (Fig. 4.1B), I found that both Mint2 N723S and Mint2 ΔPDZ mutants decrease membrane neurexin levels

compared to the wild type, suggesting that Mint2 N723S alters neurexin membrane trafficking (Fig. 4.1C).

Mint2 N723S decreases neurexin protein stability in HEK293T cells

Membrane proteins that are not correctly targeted to their proper location will eventually be degraded. To determine whether Mint2 is involved in neurexin I protein stability in the total cell lysate, I co-transfected neurexin Iα together with GFP control, GFP-Mint2 wild type, GFP-Mint2 N723S or GPF-Mint2 ΔPDZ in HEK293T cells. Total cell lysates were harvested and immunoblotted for neurexin Iα. The results revealed that Mint2 wild type increases neurexin I protein level compared to GFP control, suggesting that Mint2 stabilizes neurexin I protein expression in the total cell lysate (Fig. 4.2A). Also, Mint2 N723S decreases neurexin I protein level compared to Mint2 wild type, suggesting that the mutation destabilizes neurexin I (Fig. 4.2A). Accordingly, co-expression of neurexin with GFP-Mint2 ΔPDZ, which affects neurexin binding, decreased neurexin levels, suggesting the Mint2 binding to neurexin is critical for stability.

Also, it is important to note that the neurexin banding patterns were altered. The GFP control or Mint2 ΔPDZ mutant transfected cells have double banded neurexin Iα. In contrast, Mint2 wild type or Mint2 N723S transfected cells have single banded neurexin Iα, suggesting that there are multiple forms of neurexins. Mint2 wild type co-expression increased the levels and altered the migration of neurexin. Specifically, Mint2 wild type doubled Nrxn1α levels and transformed the two immature bands of ~160 and ~190 kDa

bands to one mature band. Together, these results suggest that not only Mint2 is involved in neurexin Iα membrane localization, but it is also involved in its maturation and processing in the Golgi.

Mint2 is enriched in the Golgi apparatus in wild type hippocampal neurons

Since Mint2 is a neuronal-specific protein, I next examined Mint2 localization in neurons. Mint-deficient hippocampal neurons were infected with Mint2 wild type, Mint2 N723S, or Mint2 ΔPDZ lentivirus at 3 DIV and immunostained for GM130 (a *cis*-Golgi marker). Although Mint2 is well established in the literature for its function at the synapse, I did not observe an obvious enrichment of Mint2 at synapses (data not shown). However, I found that Mint2 wild type is enriched in the Golgi (Fig. 4.3), confirming a previous finding (Biederer *et al.* 2002). There was no obvious difference between Mint2 wild type and Mint2 N723S in Golgi localization. However, I found that Mint2 ΔPDZ completely lost its Golgi localization compared to the wild type, suggesting that PDZ domains are essential for Mint2 Golgi localization. It is possible that Mint2 binds to a membrane protein in the Golgi, possibly via the PDZ domains.

Mint2 is enriched in the Golgi apparatus in Mint-deficient hippocampal neurons

In addition to the wild type hippocampal neurons, I also examined Mint2 Golgi localization in Mint-deficient neurons to see if the phenotype is reproducible in both neuron types. Here, Mint-deficient (Mints 1-3 deficient) hippocampal neurons were

infected with lentiviral cre recombinase together with Mint2 wild type or Mint2 ΔPDZ lentivirus at 3 DIV and immunostained for the *cis*-Golgi marker, GM130. Similar to what I found in the wild type neurons (Fig. 4.3), Mint2 wild type displayed a pronounced Golgi localization and Mint2 ΔPDZ completely lost its Golgi localization as compared to the wild type (Fig. 4.4).

Interestingly, I also observed that Mint2 Golgi localization is neuronal specific (Fig. 4.4 — see asterisk). Mint2 lentivirus also infects glial cells due to a non-neuronal specific promoter, ubiquitin. However, Mint2 does not localize to the glial Golgi (Fig. 4.4 —see arrow), further validating that Mint2 functions as a neuronal specific protein. It is possible that glial cells do not express binding partners of Mint2 in the Golgi to facilitate the Golgi localization.

Mint2 N723S decreases the kinetics and mobility at the Golgi and neuronal processes

Previous studies have shown that Mints function at the Golgi to control polarized trafficking of axonal membrane proteins in *Drosophila* (Gross *et al.* 2013). Since Mints are largely localized to Golgi (Fig. 4.3 and Fig. 4.4), I wanted to test the possibility that Mints are mobile proteins which control and traffic membrane proteins. I first performed fluorescence recovery after photobleaching (FRAP) to determine the kinetics of Mint2 protein in live primary mouse neurons that were infected with GFP-Mint2 wild type or GFP-Mint2 N723S. I found that GFP-Mint2 fluorescence signal was effectively depleted by photobleaching and partially recovered in the Golgi as would be expected for a mobile

protein (Fig. 4.5A). Indeed, 58% of GFP-Mint2 wild type recovered within 150 s, indicating that a large proportion of Mint2 wild type is mobile (Fig. 4.5B). However, the fluorescence recovery pattern was different; 58% of GFP-Mint2 wild type recovered within 150s as compared with 45% of GFP-Mint2 N723S, suggesting that N723S is less mobile in the Golgi and/or its recruitment from the cytosol is less dynamic (Fig. 4.5C). The time at 50% of the recoverable fluorescence ($\tau_{1/2}$) from GFP-Mint2 wild type and N723S at the Golgi was not different suggesting Mint2 N723S did not alter puncta turnover and stability within the Golgi.

To monitor the mobility of GFP-Mint2 puncta in neuronal processes, we used kymograph analysis to plot the fluorescence intensity of Mint2 wild type and N723S mutant. Mobile and stationary puncta could be visualized with stationary puncta seen as vertical bands and mobile puncta are indicated by diagonal bands within the kymograph (Fig. 4.5*E*). Interestingly, while the speed of the mobile Mint2 puncta was similar in wild type and N723S mutant, the percentage of immobile Mint2 puncta was greater in the N723S mutant (Fig. 4.5*F* and *G*). Overall, these data demonstrate that Mint2 is a mobile protein in neurons capable of trafficking from the Golgi to the processes; however the N723S mutant leads to reduced mobility.

Mint2 wild type co-localizes with neurexin Iα in neuronal soma and axon

Since Mint2 has a pronounced Golgi localization (Fig. 4.3 and 4.4), it would be interesting to determine whether neurexin Iα and Mint2 are co-localized in neurons, indicating that they may be trafficked together. Wild type hippocampal neurons were

transfected with GFP-Mint2 wild type and pSyn5-E-mCherry-Neurexin Iα at 5 DIV and labeled with rabbit anti-mCherry at 7 DIV. The data revealed that GFP-Mint2 wild type co-localizes with mCherry-Neurexin Iα in the soma next to the nucleus (Fig. 4.4). In addition, I also found that GFP-Mint2 wild type puncta co-localizes with mCherry-Neurexin Iα puncta in the axon, suggesting that Mint2 and neurexin Iα may traffic together in vesicles.

Mint2 N723S alters neurexin Iα axonal targeting to the synapse

Previously, I found that Mint2 N723S decreases membrane neurexin Iα compared to Mint2 wild-type in HEK293T cells (Fig. 4.2B-C). Here, I wanted to determine if this phenotype can be recapitulated in neurons. I monitored Mint2-Nrxn1a colocalization at physiological synapses formed between neurons. Primary hippocampal neurons were co-transfected with mCherry-tagged neurexin Iα and GFP-control, GFP-Mint2 wild type or GFP-Mint2 N723S at 4-5 DIV. Cultured neurons were fixed at 7 DIV, and I quantified colocalization by percentage of colabeled Nrxn Ia with GFP in axonal processes. I counted the number of GFP-Mint2 wild-type puncta and mCherry-tagged neurexin Iα puncta individually for each axon. Next, I counted how many of them are co-localized together. I observed a prominent punctate GFP signal with Mint2 wild type colocalized perfectly with the expression of mcherry-NrxnIα (Fig. 4.7A and 4.7B). The colocalization signal was not as robust when cultured neurons were transfected with GFP-Mint2 N723S.

To validate that the colocalization signal was synaptic, I performed an extensive immunofluorescence analysis using a presynaptic marker. I co-transfected wild type mouse hippocampal neurons with mCherry-tagged neurexin Iα with GFP-Mint2 wild type or GFP-Mint2 N723S at 7 or 9 DIV. Neurons were labeled with rabbit anit-mCherry polyclonal antibodies (to enhance the visualization of the mCherry signal) and rabbit anti-synaptobrevin 2 (to visualize the presynaptic terminal). The degree of co-localization of mCherry-tagged Neurexin Iα and synaptobrevin 2 indicates the number of neurexin Iα vesicle trafficked to the presynaptic terminal. The data revealed that Mint2 wild type increases the number of neurexin Iα vesicle trafficked to the presynaptic membrane compared to EGFP control (Fig. 4.7C and 4.7D), suggesting that Mint2 in involved in neurexin Iα trafficking. In addition, Mint2 N723S decreases numbers of neurexin Iα trafficked to the presynaptic membrane compared to Mint2 wild type, which is consistent with less neurexin Iα at axons (Fig. 4.7A and 4.7B).

Discussion

Autism is a neurodevelopment disorder with a strong genetic contribution. Among all the genes, disruption of neurexin I is highly implicated in autism (Kim *et al.* 2008). Neurexin I interacts directly with the PDZ domains of Mint2 (Biederer *et al.* 2002). However, it is not clear which PDZ domain directly interacts with neurexin. To determine whether an altered interaction can disrupt neurexin function, I first examined Mint2-neurexin I interaction in HEK293T cells. I did not find any binding deficit

between Mint2 N723S mutant and neurexin I compared to the wild type in GST pull down experiments (Fig. 4.1A). However, I found that Mint2 N723S significantly decreases neurexin I membrane localization in HEK293T cells (Fig. 4.1B-C). Moreover, Mint2 ΔPDZ mutant also decreased neurexin I membrane localization compared to Mint2 wild type (Fig. 4.1B-C). It is interesting to note that Mint2 ΔPDZ mutant binds neurexin less efficiently and therefore does not traffic neurexin properly. In contrast, Mint2 N723S binds to neurexin normally, but still alters neurexin I trafficking. Therefore, Mint2 ΔPDZ and Mint2 N723S alter neurexin I membrane localization through two different mechanisms. One possible explanation is that Mint2 N723S binds to neurexin, but it does not release neurexin to the membrane. This explanation suggests that the cargo must be somehow mislocalized or degraded, confirming what I found in both HEK293T cells (Fig. 4.1 and 4.2) and neurons (Fig. 4.7).

It is also interesting to note that neurexin Iα in the total cell lysate have different banding patterns (Fig. 4.2A). The GFP control or GFP-Mint2 ΔPDZ mutant transfected cells have double-banded neurexins. In contrast, GFP-Mint2 wild type or GFP-Mint2 N723S transfected cells only display single-banded neurexins. Together, these results indicate that there are two different species of neurexins, suggesting that different post-translational modifications are involved. A membrane protein such as neurexin I is first translated in the endoplasmic reticulum. Posttranslational modification starts in the endoplasmic reticulum and continues in the Golgi. Since Mint2 is a neuronal adaptor protein localized to the neuronal Golgi apparatus, it is possible that it shuttles its cargo between Golgi compartments for posttranslational modification. Membrane proteins that

are not properly shuttled by Mint2 would not be properly modified and possibly mis-localized. Therefore, Mint2 may be involved in the posttranslational modification of neurexin.

A function of Mint2 in protein trafficking is not surprising. In vertebrate neurons (Biederer *et al.* 2002) and *C. elegans*, Mint 1 is concentrated at the Golgi apparatus and localized throughout axons and dendrites, strongly suggesting that Mint1 is involved in protein trafficking. The PDZ domains of Mint1 have been shown to traffic NMDA receptor subunits to the postsynaptic membrane by binding to Kif17, a kinesin motor protein (Setou *et al.* 2000). Similar to Mint1, Mint2 also has a pronounced Golgi localization (Fig. 4.3 and 4.4). Since Mints 1 and 2 are highly conserved at the C-terminus, Mint2 could, in principle, interact with Kif17 or other kinesin proteins to traffic neurexin I to the membrane. The trafficking complex between Mint2 and neurexin involves other motor proteins or adaptors. It would be interesting to identify the players in the trafficking complex, as it could reveal novel interactions.

Previously, Mint2 has been shown to be concentrated in the *trans*-Golgi complex with TGN38 (a *trans*-Golgi marker) labeling in cultured hippocampal neurons doubled stained with antibodies against endogenous Mint2 and *trans*-Golgi (Biederer *et al.* 2002). I found that Mint2 is also located in the *cis*-Golgi complex with GM130 (a *cis*-Golgi marker) labeling in cultured hippocampal neurons infected with GFP-Mint2 wild type and labeled with antibody against endogenous *cis*-Golgi (Fig. 4.3 and 4.4). Trans-Golgi localization indicates the involvement in membrane transport because it is physically closer to the cell membrane. However, the functional role of the *cis*-Golgi localization of

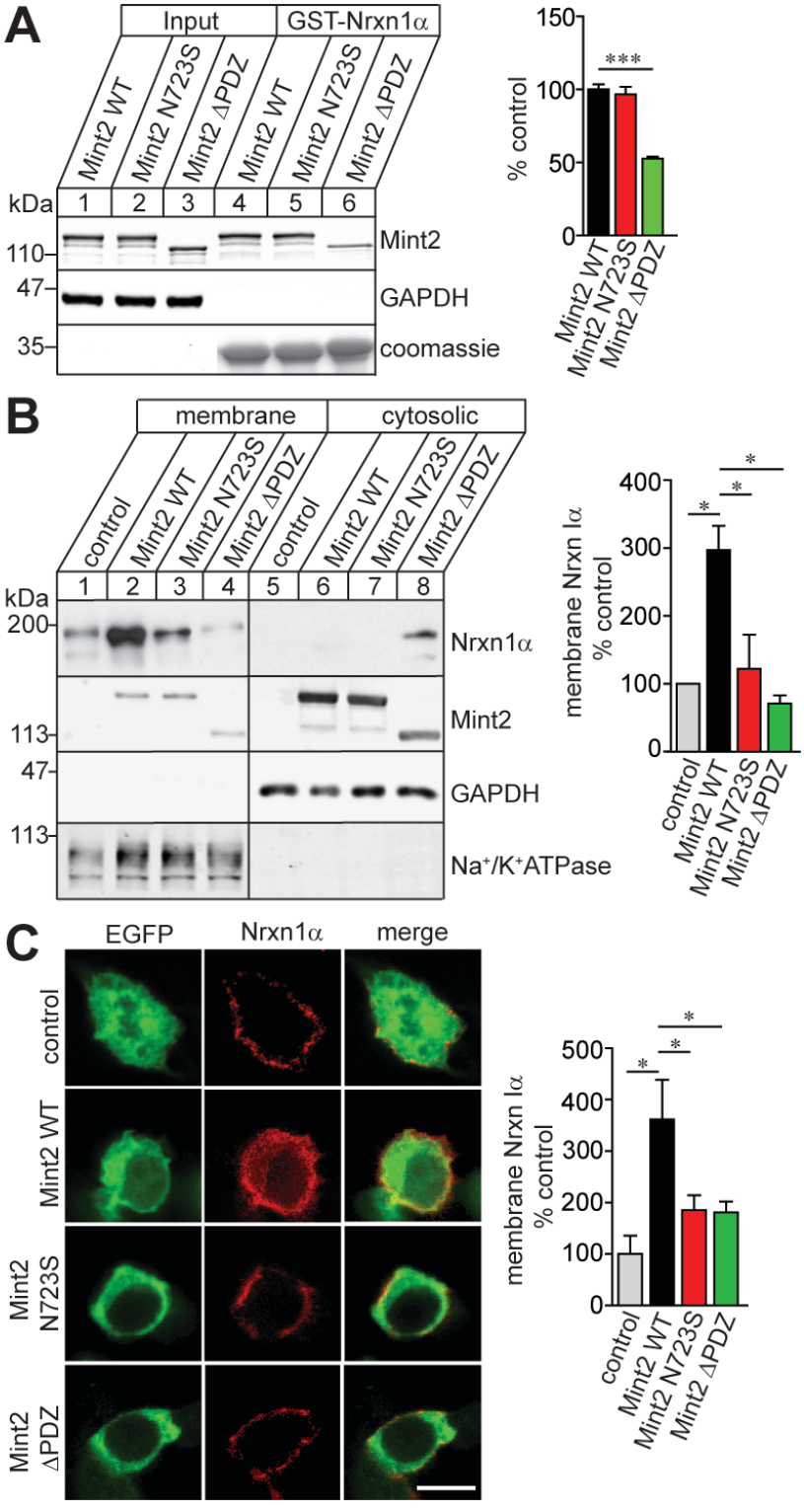
Mint2 is not clear. Mint2 could shuttle membrane proteins from the *cis*-Golgi to *trans*-Golgi or from the ER to the *cis*-Golgi. However, since Mint2 is not localized in the ER, the ER to the *cis*-Golgi shuttling is unlikely. Also, Mint2 may carry out specialized functions in different Golgi compartments.

Mint2 Golgi localization is not a novel finding. What is really interesting is that Mint2 Golgi localization is PDZ domain dependent, which is a novel finding. The PDZ domains are involved in Mint2 Golgi localization in both wild-type neurons (Fig. 4.3) and Mint-deficient neurons (Fig. 4.4), strongly suggesting that the PDZ domains target Mint2 to the Golgi. A plausible mechanism is that the PDZ domains bind to other proteins and recruit Mint2 to the Golgi. It is important to note that Mint2 Golgi localization is not visible in HEK293T cells if Mint2 was transfected alone (Fig. A1.2), suggesting that other proteins (e.g., neuronal specific adaptor proteins) are involved in Mint2 Golgi targeting that are only present in neurons.

In neurons, Mint2 N723S decreases neurexin I localization in axons (Fig. 4.7A-B) and presynaptic terminals (Fig. 4.7C-D) compared to Mint2 wild type. Mint2 N723S also showed a decrease in FRAP of Golgi compared to Mint2 wild type (Fig. 4.5). Together, these data suggest that Mint2 N723S is more trapped in the Golgi and therefore not able to move neurexin to presynaptic terminals efficiently.

Figure 4.1: Mint2 N723S decreases neurexin Iα membrane trafficking in HEK293T cells.

A, Pull down experiment of HEK293T cells transfected with Mint2 wild type or Mint2 N723S mutation on immobilized GST-neurexin I (Nrxn I) fusion protein. Coomassie stained gel of GST-Nrxn I. ***B,*** Quantification of Mint2 wild type or mutants pulled down by GST-neurexin I. ***C***, Mint2 N723S decreases neurexin I trafficking to the membrane. HEK293T cells co-transfected with neurexin Iα (Nrxn Iα) together with GFP control, GFP-Mint2 wild type (WT), GFP-Mint2 N723S or GFP-Mint2 ΔPDZ were subjected to membrane and cytosolic fractionation and blotted with antibody against Nrxn I and Mint2. Na^+/K^+ ATPase and GAPDH were used as a membrane and cytosolic marker, respectively. ***D,*** Quantification of the effect of Mint2 N723S mutant on Nrxn Iα membrane protein level. ***E,*** Representative images of HEK293T cells transfected with mCherry-Neurexin Iα together with EGFP control, GFP-Mint2 wild type, GFP-Mint2 N723S, or GFP-Mint2 ΔPDZ. Cell surface mCherry-Neurexin Iα signal was amplified with rabbit anti-mCherry antibody without detegent permeabilization. ***F,*** Quantification of the effect of Mint2 mutants on cell surface mCherry-Neurexin Iα. *$p<0.05$; **$p<0.010$; ***$p<0.005$.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Input
GST-Nrxn1α
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Mint2 N723S
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47
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100
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Mint2 N723S
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Mint2 N723S
Mint2 ΔPDZ

Figure 4.2: Mint2 N723S decreases neurexin Iα protein stability in HEK293T cells.

A, HEK293T cells were co-transfected with neurexin Iα (Nrxn Iα) together with GFP control, Mint2 wild type, Mint2 N723S, or Mint2 ΔPDZ mutant. Total cell lysates were harvested and immoblotted with antibodies against Nrxn I, Mint2, and GAPDH. GAPDH was used a loading control in the same blot. ***B,*** Effect of Mint2 mutants on Nrxn Iα total protein expression. *p<0.05; **p<0.010; ***p<0.005.

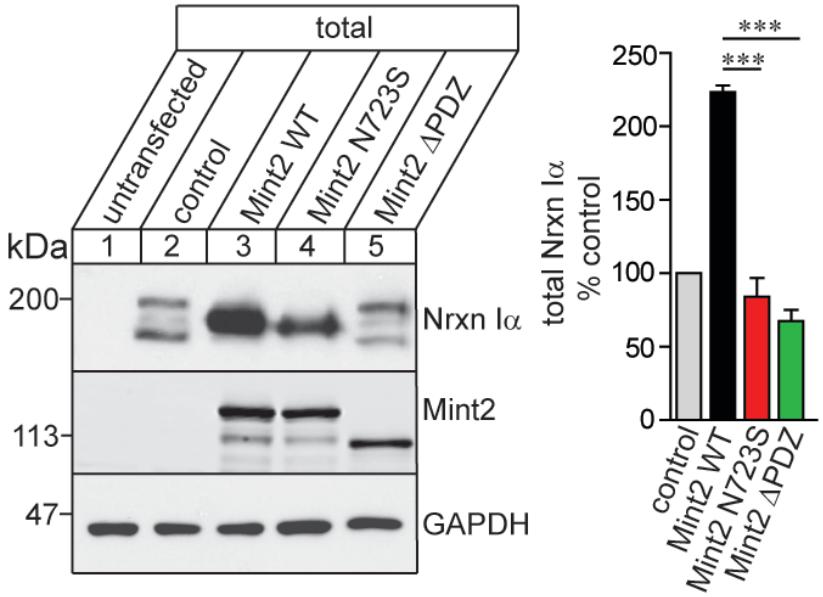


Figure 4.3: Mint2 Golgi localization in wild type hippocampal neurons is PDZ domain dependent.

Representative images of wild-type hippocampal neurons infected with GFP-Mint2 wild type (WT), GFP-Mint2 N723S, or GFP-Mint2 ΔPDZ. Neurons were stained with GM130 (a Golgi maker) at 14 DIV. Both Mint2 WT and N723S co-localize with Golgi. The lower panel shows that Mint2 ΔPDZ mutant loss its Golgi localization.

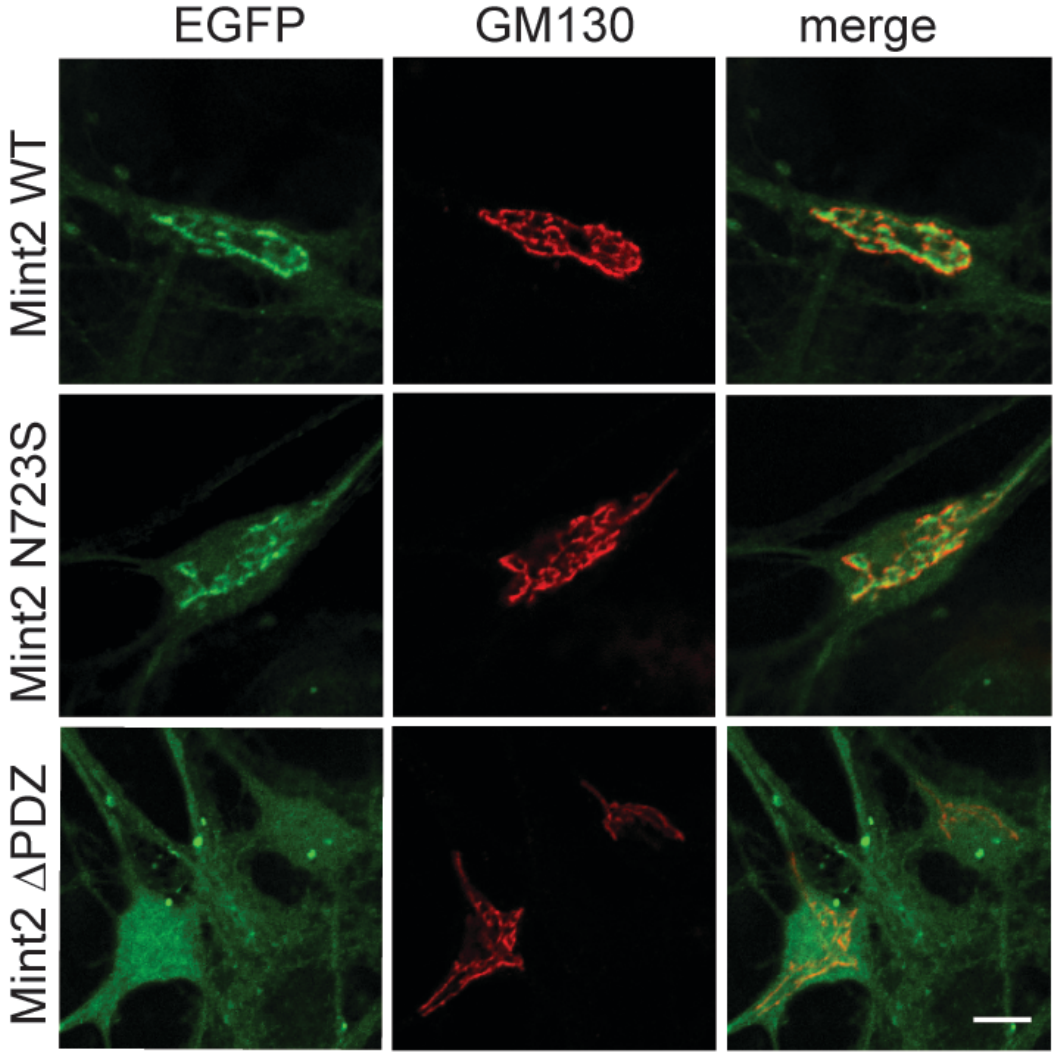


Figure 4.4: Mint2 Golgi localization in Mint-deficient hippocampal neurons depends on the PDZ domains.

Representative images of Mint-deficient hippocampal neurons infected with GFP-Mint2 wild type (WT) or GFP-Mint2 ΔPDZ lentivirus were labeled with GM130 (a *cis*-Golgi marker) at 14 DIV. The upper panel shows that Mint2 WT (green) co-localizes with Golgi (red). The asterisk indicates a neuron with Golgi localization. The arrow shows that a glial cell without Golgi localization, suggesting that Mint2 Golgi localization in neuronal-specific. The lower panel shows that Mint2 ΔPDZ mutant does not localized to the Golgi, suggesting that PDZ domains are required for Mint2 Golgi localization. Note that due to usage of GFP-tagged cre recombinase lentivirus in Mint-deficient hippocampal neurons, the nuclei shown here are in color green.

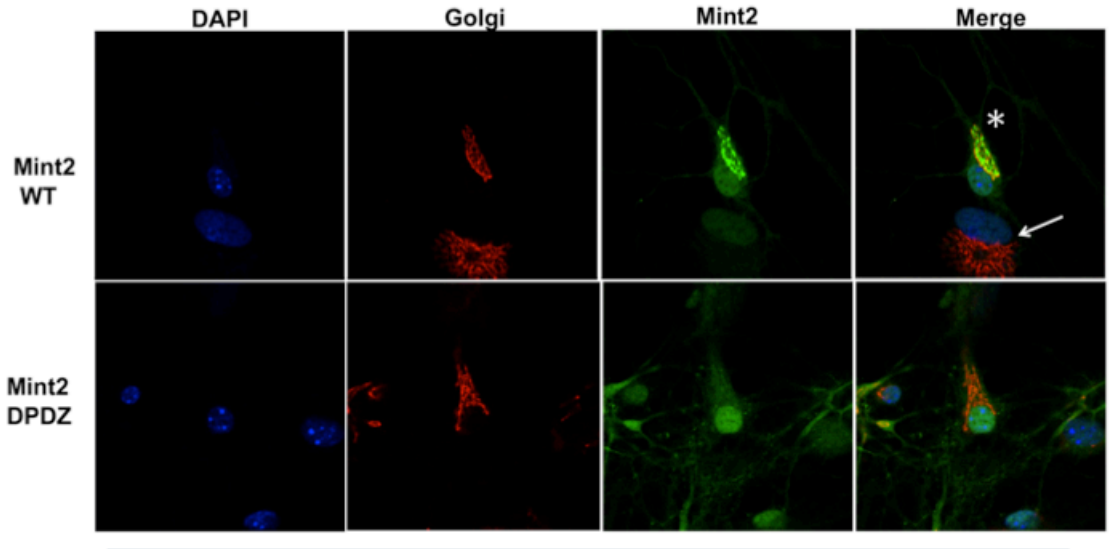
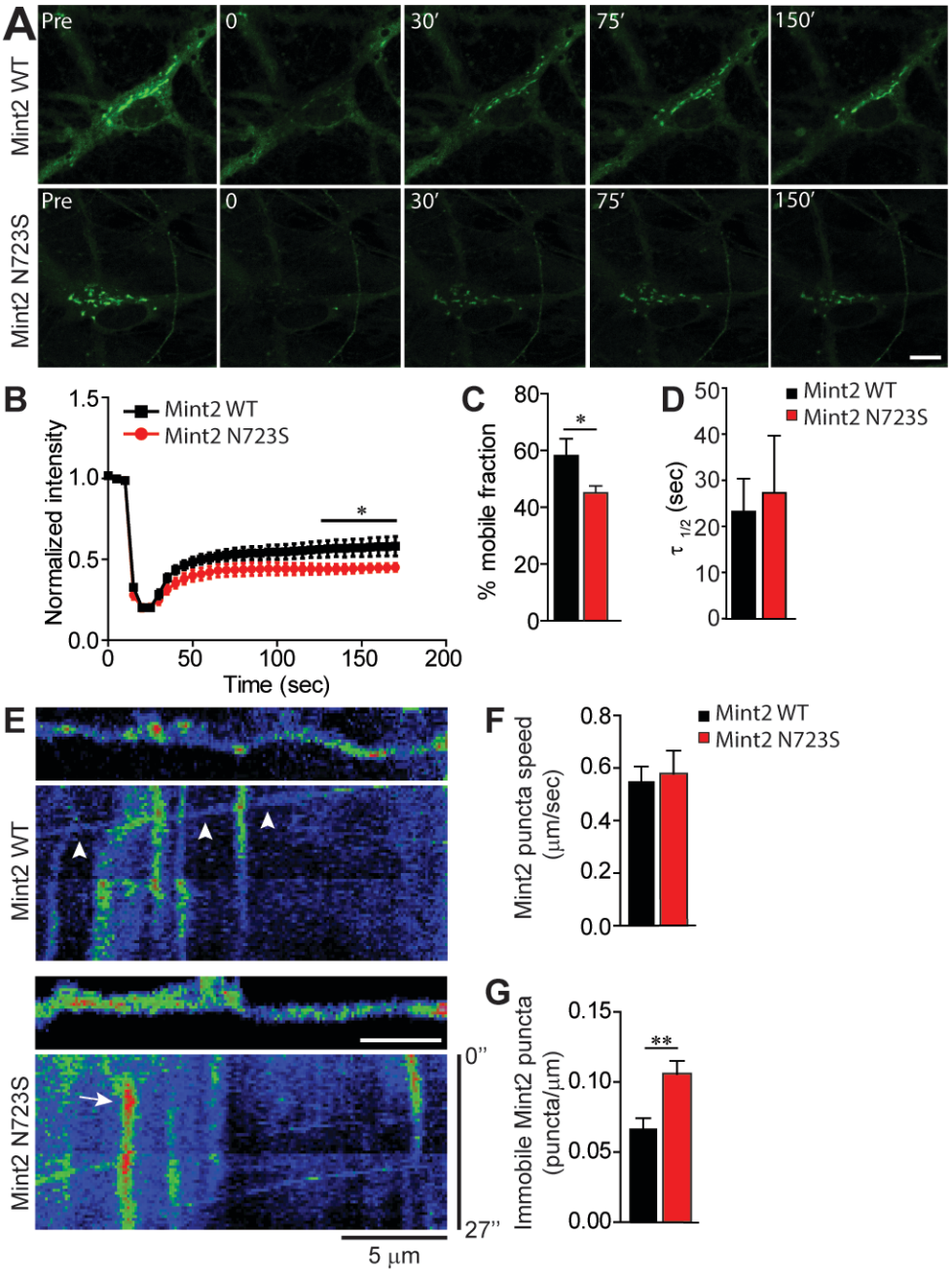


Figure 4.5: Mint2 N723S is less mobile in Golgi in wild type hippocampal neurons.

A, FRAP imaging of GFP-Mint2 wild type and N723S in the Golgi. Regions encompassing Golgi were photobleached using 3 passes (15 s total) of 100% laser power, which reduced the initial fluorescence intensity to 25%. Recovery fluorescence was acquired with 1% laser power and imaged every 5 s for 150 s. ***B,*** Normalized intensity of GFP-Mint2 wild type and N723S signal during fluorescence recovery after photobleaching. ***C,*** Bar graph shows the percentage of Mint2 wild type or N723S that is mobile in the Golgi. *$p<0.05$; **$p<0.010$; ***$p<0.005$.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27″
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Mint2 WT
Mint2 N723S
Mint2 puncta speed (μm/sec)
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Immobile Mint2 puncta (puncta/μm)

Figure 4.6: Mint2 wild type co-localizes with Neurexin Iα in neuronal soma and axon.

Representative images of wild type hippocampal neurons transfected with GFP-Mint2 wild type (WT) and mCherry-Neurexin Iα at 5 DIV. Neurons were stained with rabbit anti-mCherry (to visualize and amplify the mCherry signal) at 7 DIV. GFP-Mint2 WT (green) co-localizes with mCherry-Neurexin Iα (red) and the merge images are displayed in yellow.

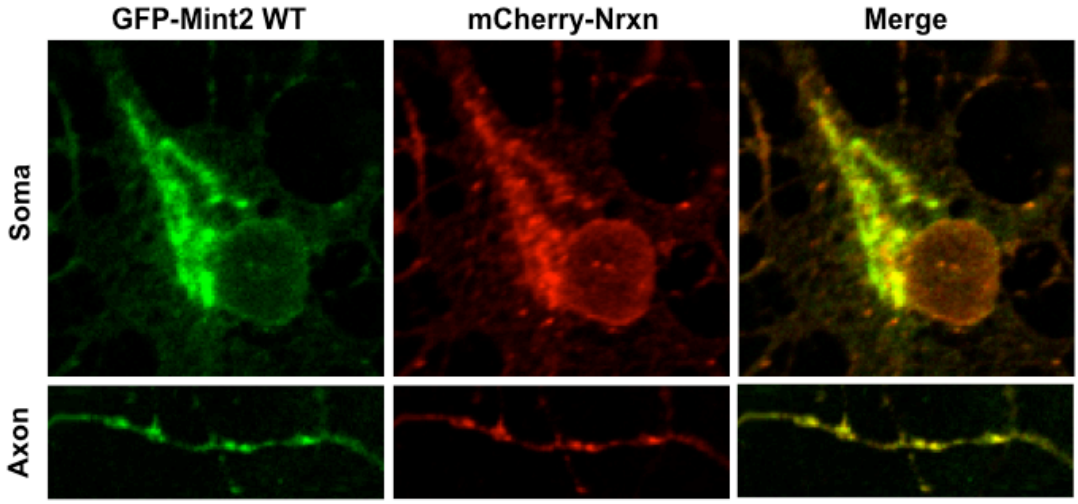
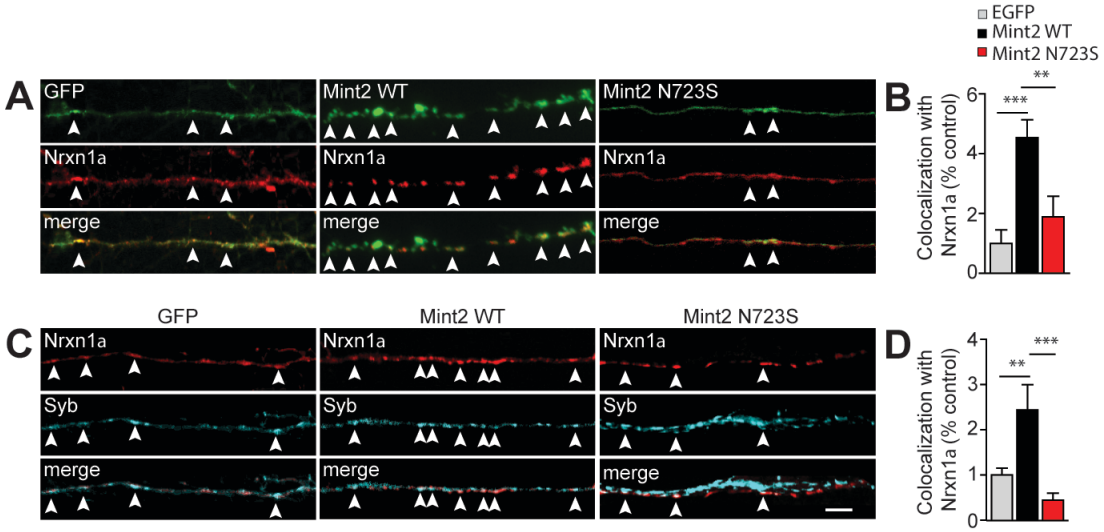


Figure 4.7: Mint2 N723S decreases Neurexin Iα axonal targeting to the synapse.

A, Representative images of axonal segments of wild type hippocampal neurons transfected with mCherry-Neurexin Iα together with EGFP control, GFP-Mint2 wild type or GFP-Mint2 N723S at 5 DIV. Neurons were labeled with rabbit anti-mCherry antibody at 7 DIV. ***B,*** Quantification of mCherry-Neurexin Iα and GFP colocaliztion. Data are means ± SEM. Differences were determined by one-way ANOVA. Cells were collected from a total 30 axons from 3 independent cultures. Scale bars, 5 μm. ***C,*** Representative images of wild type hippocamapl neurons transfected with mCherry-Neurexin Iα together with EGFP control, GFP-Mint2 wild type or GFP-Mint2 N723S at 7 DIV and immunostained for mouse anti-synaptobrevin at 9 DIV. Numbers in bars represent analyzed axons from 3 neuronal cultures (EGFP=14 cells, WT=38 cells, N723S=32 cells). ***D,*** Quantification of mCherry-Neurexin Iα and synaptobrevin (Syb) colocaliztion. Data are means ± SEM. Differences were determined by one-way ANOVA. Numbers in bars represent analyzed neuronal processes from 1 neuronal culture (EGFP=23 processes, WT=16 processes, N723S=21 processes). Scale bars, 10 μm. *p<0.05; **p<0.010; ***p<0.005.



CHAPTER FIVE: Mint2 N723S and CNV alter excitatory synaptogenesis and synaptic transmission

Introduction

The human brain has billions of neurons and each neuron is capable of generating 1,000 to 10,000 synapses. Synapses are specialized asymmetric intercellular contacts between neurons that function in neurotransmission. During neurotransmission, neurotransmitters are released from the presynaptic terminal into the synaptic cleft and bind to the receptors on the postsynaptic membrane. In order for the brain to function properly, signals from neuron to neuron must be carried out with both spatial and temporal precision.

Our laboratory has shown that Mint2 overexpression contributes to synaptic dysfunction (Sullivan *et al.* 2014). Mint2 overexpression in wild type neurons increases miniature event frequency and amplitude in excitatory synapses compared to control neurons, suggesting that Mint2 overexpression alters quantal size, quantal frequency, synapse number, and/or postsynaptic receptor numbers. Moreover, acute knockout of all three Mints in cultured neurons decreases spontaneous neurotransmitter release at excitatory synapses, which can be rescued with Mint2 wild type (Ho *et al.* 2006, Sullivan *et al.* 2014). Together, these data strongly suggest that Mint2 is involved in spontaneous excitatory neurotransmission.

Synaptogenesis, the generation and maturation of synapses, is mediated by cell adhesion proteins, neurexins and neuroligins (Serafini 1999, Yamagata *et al.* 2003).

Synaptogenesis has two steps. First, the initial contact between the axonal growth cone and a postsynaptic target must be established. Second, the assembly of presynaptic and postsynaptic proteins at the contact site leads to the maturation and specification of the synapse.

In the previous chapter, I found that Mint2 N723S alters neurexin I membrane localization in HEK293T cells (Fig. 4.1B-C). In neurons, Mint2 N723S also decreases neurexin I axonal targeting to the synapses (Fig. 4.7). Based on these findings, I hypothesized that Mints N723S would lead to decreased synapse formation. In this chapter, I examined the effect of Mint2 N723S in synapse formation by performing heterologous synapse formation assay. In addition, I also examined the effect of Mint2 N723S on spontaneous excitatory neurotransmission to determine whether an altered synaptogenesis leads to an altered neurotransmission. To correlate function to form, lastly, I examined synapse ultrastructure to determine whether Mint2 N723S caused any structural deficit of the synapse. I also examined Mint2 copy number variant, with the exception that the electrophysiological analysis was previously published (Sullivan, 2014).

Results

Mint2 N723S mutant decreases synaptogenesis

To determine whether Mints are functional in synaptic formation dependent on neurexin-neuroligin (Nrxn-NL) binding, I used a neuron-fibroblast co-culture approach where it has been shown that NL-1 expressed in non-neuronal cells such as COS-7 cells

induces the formation of presynaptic specifications in contacting neuronal axons (Scheiffele *et al.* 2000). I infected Mint-deficient hippocampal neurons with Mint2 wild type, Mint2 N723S, Mint2 ΔPDZ, or heterozygote (+/N723S) lentiviruses. At DIV 10, I seeded COS-7 cells transfected with mVenus-NL-1 to induce synapse formation indicated by presynaptic synapsin staining that was clustered on NL-1 expressing COS-7 cells. I found that neurons lacking Mints (Mint KO) impaired synapse formation compared to wild type neurons (Fig. 5.1A), suggesting that Mint2 is involved in synapse formation. Infection of lentiviral Mint2 wild type was able to rescue heterologous synapse formation in Mint-deficient neurons. Also, I found that Mint2 N723S, ΔPDZ, or heterozygous +/N723S were not able to rescue the presynaptic clustering of synapsin in Mint-deficient neurons suggesting the N723S mutant may not efficiently transport neurexin to the presynaptic membrane to rescue heterologous synapse formation. Moreover, these results suggest that neurexin membrane transport requires the Mint2 PDZ domains.

Mint2 N723S mutant decreases spontaneous neurotransmitter release

To determine whether the morphological changes in neuronal growth (Fig. 3.3A) and synapse numbers in Mint2 N723S (Fig. 5.1A) neurons lead to functional alterations in synaptic transmission, I examined the effect of the Mint2 N723S mutation on excitatory synaptic transmission by whole-cell voltage-clamp recordings to measure the frequency and amplitude of spontaneous miniature events from Mint-deficient (*M1*$^{-/-}$*; fM2/fM2; M3*$^{-/-}$) hippocampal neurons treated with lentiviral cre recombinase alone (KO),

cre recombinase and rescue with Mint2 wild type, Mint2 N723S, or Mint2 ΔPDZ lentivirus. I found that consistent with the previous studies (Ho *et al.* 2006), Mint-deficient neurons (Mint2 KO) showed a reduction in "mini" frequency in excitatory synapses compared to Mint2 wild type-infected neurons. In addition, Mint2 N723S mutant caused a 46% decrease of "mini" frequency in excitatory synapses compared to Mint2 wild type, suggesting that N723S mutation decreases the frequency of quantal release or synapse density (Fig. 5.2A). In addition, I found that Mint2 ΔPDZ and Mint2 KO neurons showed a similar decrease in "mini" event frequency compared to the wild type (Fig. 5.2A), suggesting that PDZ domains are involved in neurotransmission in the presynaptic terminal. However, "mini" amplitudes were not changed between all treatment groups (Fig. 5.2A), indicating that Mint2 mutations did not change the quantal size or postsynaptic receptor number.

To further validate whether heterozygous Mint2 N723S alters spontaneous excitatory synaptic transmission, I used whole-cell voltage-clamp recordings to measure the frequency and amplitude of spontaneous miniature events from wild type hippocampal neurons treated with lentiviral Mint2 wild type or N723S mutant. Consistent with what I found in Mint-deficient neurons (Fig. 5.2A), there was a similar 48% reduction of "mini" frequency and no change in "mini" amplitude with Mint2 N723S as compared to the wild type, suggesting that Mint2 N723S could be a dominant negative mutation (Fig. 5.2B). Overall, the results from both Mint knockout background (Fig. 5.2A) and wild type background (Fig. 5.2B) strongly indicate that Mint2 N723S

impairs spontaneous excitatory synaptic transmission by decreasing quantal release frequency or synapse density.

Mint2 N723S mutant does not alter synapse ultrastructure

To correlate synapse structure with the functional changes I found in synaptogenesis (Fig. 5.1A) and synaptic transmission (Fig. 5.2A), I examined the structure of asymmetric excitatory synapses in Mint-deficient hippocampal neurons infected with cre recombinase and rescued with Mint2 wild type or Mint2 N723S lentivirus by quantitative electron microscopy. However, I found no significant changes of Mint2 N723S as compared to Mint2 wild type, suggesting that N723S does not alter the synapse structure of asymmetric synapses (Table. 5.3). A previously study has shown no obvious difference between control and Mint deficient neurons in asymmetric synapses (Ho *et al.* 2006), suggesting that Mint2 does not have a significant role in synapse structure. Also, I examined the structure of symmetric inhibitory synapses and found no difference between Mint2 wild type and Mint2 N723S (Table. 5.4).

Mint2 CNV promotes synaptogenesis

In the previous chapters, I found that Mint2 overexpression increases neurexin membrane localization in HEK293T cells (Fig. 3.1B-C) and neurexin synapse localization compared to the EGFP control (Fig. 4.7). Here, I determined whether Mint2 overexpression also promotes synapse formation in a heterologous synapse formation

assay with wild type neurons. COS-7 cells expressing NL1-mVenus fusion protein as a postsynaptic target were co-cultured with neurons infected with lentiviral control (wild type) or Mint2 overexpression (Mint2 OE). I found that Mint2 overexpression significantly increases synapse formation by 60% compared to the wild type control (Fig. 5.3), suggesting that Mint2 copy number variant promotes synapse formation.

Mint2 CNV promotes vesicle trafficking and docking in excitatory synapses

Previously, our laboratory has shown that Mint2 overexpression in wild type neurons increases spontaneous excitatory neurotransmission (Sullivan *et al.* 2014). Next, to correlate the changes in function to the changes in form, I determined whether Mint2 copy number alters synaptic structure by quantitative electron microscopy of the synapses from cultured wild type neurons treated with either a lentiviral control or Mint2 wild type by quantitative electron microscopy (Fig. 5.4). There were no obvious differences between control and Mint2 wild type infected neurons in presynaptic area, perimeter, length of postsynaptic density, and vesicle numbers 150 nm away from the active zone (Table 5.1). However, I found that Mint2 wild type infected neurons had significant increase of the total vesicles per terminal by 30% as compared to the control (Fig. 5.4B), suggesting that Mint2 copy number variant may enhance vesicular trafficking to the presynaptic terminal. Also, I found out that Mint2 wild type infected neurons significantly increases the number of docked vesicles per active zone by 30% (Fig. 5.4B), suggesting that Mint2 CNV indirectly promote vesicle docking and vesicular release by trafficking more vesicles to the presynaptic terminal. This data is consistent with the

previous finding showing that Mint2 overexpression increases the frequency of spontaneous neurotransmitter release compared to the control (Sullivan *et al.* 2014). Overall, these data support that (1) Mint2 acts as an important regulator of presynaptic neurotransmitter release machinery (Biederer and Südhof 2000, Ho *et al.* 2006), and (2) Mint2 CNVs promote vesicular trafficking and vesicle docking, which may contribute to the imbalance in neurotransmission that is associated with autism.

I also examined the symmetric (presumptive inhibitory) synapses and found no significant differences between the wild type control and Mint2 overexpression (Table 5.2), further validating Mint2's specific role in excitatory synapses.

Discussion

Effects of Mint N723S on synapse formation and synaptic function

Neurexins and neuroligins are presynaptic and postsynaptic cell adhesion molecules that have been associated with synapse formation. I examined whether the reduced neurexin membrane trafficking of Mint2 N723S found in HEK293T cells (Fig. 3.1B-C) and in neurons (Fig. 4.7) decreases synapse formation. The present data shows that Mint2 N723S reduces synapse formation in heterologous synapse formation assay similar to the knockout (Fig. 5.1A), suggesting that N723S may behave like a loss-of-function mutation. Also, Mint2 +/N723S heterozygote neurons exhibit reduced synapse formation similar to Mint2 knockout and Mint2 N723S neurons (Fig. 5.1A), suggesting that Mint2 N723S may be a dominant negative mutation.

To correlate the reduction in synapse formation with an altered functional change, I next examined whether Mint2 N723S alters excitatory synaptic transmission since Mint2 is preferentially localized in excitatory neurons (Ho *et al.* 2006). I found that while N723S does not change the "mini" amplitude, it decreases the "mini" frequency similar to the knockout (Fig. 5.2A). This same effect was also observed in Mint2 KO and ΔPDZ mutants. Together, these data suggest that (1) Mint2 N723S is a loss-of-function mutation, that only effects the presynaptic neurotransmitter release machinery, and (2) Mint2 overexpression is a gain-of-function mutation, that effects both presynaptic and postsynaptic neurotransmission (Sullivan *et al.* 2014).

Effect of Mint2 copy number variant on synapse formation and synaptic function

As a preliminary test, I examined the effects of Mint2 overexpression on the total protein levels of PSD95 and synapsin by quantitative immunoblotting. I found no difference between Mint2 wild type and Mint2 overexpression (data not shown), suggesting that Mint2 overexpression did not increase the total synapse number, which is consistent with previous *in vivo* work demonstrating that Mint2 knockout neurons did not alter synaptic protein expression significantly (Ho *et al.* 2006). However, since Mint2 overexpression alters spontaneous excitatory neurotransmission by increasing both the "mini" frequency and "mini" amplitude (Sullivan *et al.* 2014), one would expect Mint2 overexpression could increase synapse density. If there was no change in synapse density, the higher "mini" frequency was caused by the increased in vesicle density and vesicular release at the presynaptic terminal (Fig. 3.3B).

Alternatively, Mint2 overexpression is involved in the specialized synapse and not in the global regulation of synaptic proteins. One could examine the effect of Mint2 overexpression for excitatory synapses since Mint2 overexpression alters spontaneous excitatory neurotransmission (Sullivan *et al.* 2014).

Finally, I examined heterologous synapse formation assay to reveal an effect of Mint2 on synaptogenesis since the co-cultured system offers less potentially compensatory mechanisms as compared to the wild type culture. The present data show that Mint2 overexpression increases synaptogenesis compared to the wild type control (Fig. 5.3B), suggesting that Mint2 overexpression is a gain-of-function mutation. Together, the data indicate that (1) Mint2 is involved in synapse formation by trafficking neurexins to the presynaptic terminal and (2) Mint2 overexpression promotes more synapse formation by trafficking more neurexins to the presynaptic terminal (Fig. 4.7).

The increase in synaptogenesis (Fig. 5.3) correlates well with the increase in excitatory spontaneous neurotransmission (Sullivan *et al.* 2014). Overexpression of Mint2 increases the frequency of miniature excitatory postsynaptic current (mEPSC), suggesting that Mint2 copy number variant increases the frequency of presynaptic release probability. Mint2 copy number variant also increases the amplitude of mEPSC, suggesting that Mint2 copy number variant increases the postsynaptic receptor density. Together, these data suggest that Mint2 overexpression has both presynaptic and postsynaptic effects, which is different from the Mint2 knockout and Mint2 N723S mutant, which only effect the presynaptic release probability (Fig. 5.2).

Next, I determined whether the increased in excitatory spontaneous neurotransmission (Sullivan *et al.* 2014) correlates with any structural changes of the synapse. I found that Mint2 copy number variant significantly increases the total vesicles per terminal and the number of docked vesicles per active zone by 30% (Fig. 5.4B). These data strongly support that Mint2 copy number variant promotes vesicle trafficking to the presynaptic terminal. However, it does not imply that Mint2 copy number variant promotes vesicle docking and releasing, at least not directly. This is because since the increases in both measurements (total vesicles per terminal and the number of docked vesicles per active zone) is the same (30%) (Fig. 5.4B), hence, the increased in docked vesicles comes from the similar increased in vesicles concentration. In sum, Mint2 copy number variant promotes more vesicle trafficking to the presynaptic terminal, and therefore, more vesicles are available for docking and releasing.

Autism is associated with an imbalance of excitatory and inhibitory inputs. The present data suggest that Mint2 N723S and Mint2 ΔPDZ mutant decrease the frequency of excitatory transmission and may alter the precise balance between excitation and inhibition. Based on the established model, Mint2 PDZ domains interact with neurexin at the presynaptic terminal to facilitate vesicular fusion (Biederer and Südhof 2000). However, this model does not fully explain my data. Mint2 ΔPDZ mutant lacks interaction with neurexin (Fig. 3.1A), and therefore, it also reduces the frequency of spontaneous excitatory transmission (Fig. 5.2). However, Mint2 N723S mutant exhibits no binding deficit to neurexin (Fig. 3.1A), but it still reduces the release frequency (Fig. 5.2). This suggests that Mint N723S alters synaptic transmission by a mechanism that

may be independent of binding to neurexin or at least not fully depend on its interaction with neurexin. A possible explanation for this discrepancy is that Mint2 N723S binds to neurexin normally, but traffics less neurexin to the synapses. A decrease in synapse formation may be an explanation for a decrease in neurotransmission, which suggests a lower synapse density that is responsible for the lower "mini" frequency.

Autism spectrum disorder is a neurodevelopmental disorder that is characterized by impaired social interaction, aberrant language acquisition, and repetitive behavior. An abnormal neuronal connectivity that leads to perturbation in information processing may cause autism (Herbert 2005, Polleux and Lauder 2004). The disorder is diagnosed strictly on behavioral criteria and impaired social interaction is one of the key symptoms of autism. It would be interesting to examine neurexin Iα or Mint2 knockout mice to see whether they have behavioral phenotypes that resemble the core symptoms found in autism. Neurexin Iα knockout mice displayed reduced social interaction and spontaneous locomotor activity in novel environment (Grayton *et al.* 2013). Similarly, Mint2 knockout mice displayed decreased intruder exploration behavior in a resident intruder test and lessen interest to attractive stimuli compared to wild type mice (Sano *et al.* 2009). In addition, *in vitro* studies have shown that neurexin or Mint2 deficiency all lead to decrease in spontaneous neurotransmission (Missler *et al.* 2003, Kattenstroth *et al.* 2004, Etherton *et al.* 2009). An impaired neurotransmission can cause a network-wide disruption in the brain and behavioral abnormalities that are found in autism. Here, I propose that Mint2 copy number variant and Mint2 N723S mutation cause aberrant

synapse formation and disrupt neurotransmission as plausible mechanisms that contribute to autism.

Figure. 5.1: Mint2 N723S mutant alters synaptogenesis.

A, Mint2 N723S mutation decreases synaptogenesis in Mint-deficient (*M1*$^{-/-}$*; fM2/fM2; M3*$^{-/-}$) hippocampal neurons. Representative images of COS-7 cells expressing NL1-mVenus fusion protein (green) that were cocultured with neurons to induce synapse formation (indicated by synapsin labeling in red). The degree of synapse formation was determined by the co-localization of the presynaptic marker synapsin (red) with the postsynaptic NL1-mVenus (green). ***B,*** Quantification of synapse formation of all Mint2 mutants compared to the wild type. *p<0.05; *p<0.010; ***p<0.005.

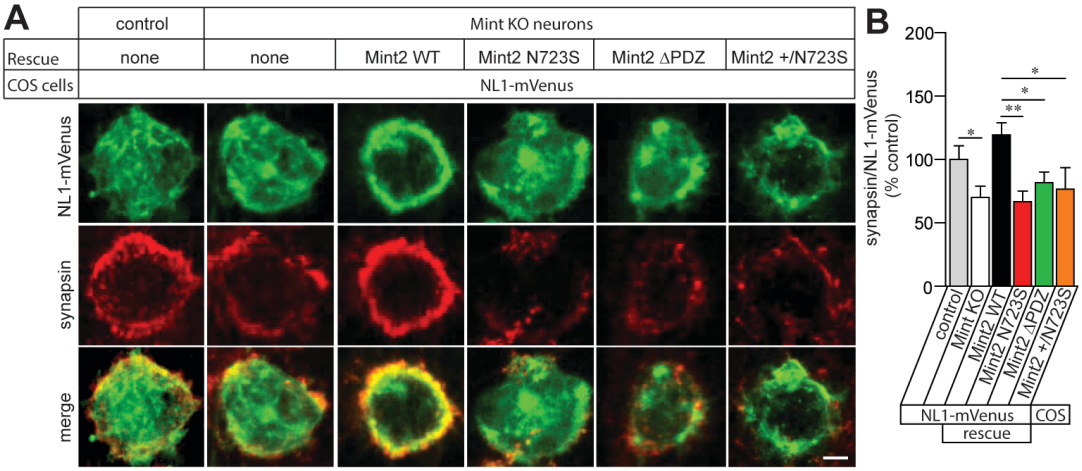


Figure. 5.2: Mint2 N723S mutation decreases excitatory spontaneous neurotransmitter release in both Mint-deficient and wild type neurons.

A, Sample traces showing mEPSC of control, Mint2 KO, Mint2 wild type, Mint2 N723S, or ΔPDZ mutants in Mint-deficient (*M1*$^{-/-}$*; fM2/fM2; M3*$^{-/-}$) hippocampal neurons. Bar graphs showing that Mint2 KO, N723S, and ΔPDZ mutants significantly decrease the frequency of mEPSC compared to the wild type. The mEPSC amplitude does not change significantly in all treatments (Control, n=9, WT, n=15; KO, n=9; N723S, n=13; ΔPDZ, n=12; *$p<0.05$). ***B,*** Sample traces showing mEPSC of Mint2 wild type or Mint2 N723S mutant in wild type hippocampal neurons. Bar graphs showing that Mint2 N723S mutant significantly decreases the frequency of mEPSC compared to the wild type. The mEPSC amplitude does not change between the two treatments (WT, n=12; N723S, n=5; *$p<0.05$).

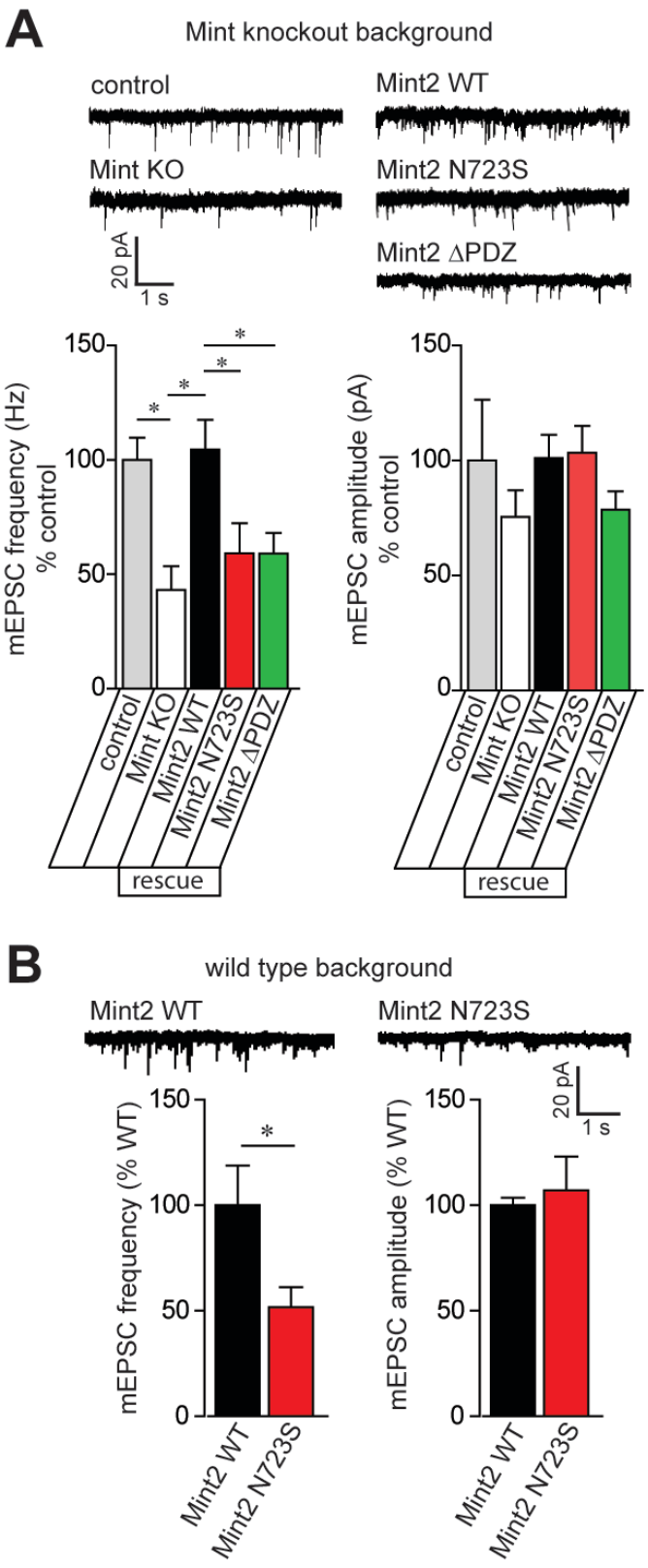

A
Mint knockout background
control
Mint KO
Mint2 WT
Mint2 N723S
Mint2 ΔPDZ
20 pA
1 s
mEPSC frequency (Hz)
% control
mEPSC amplitude (pA)
% control
150
100
50
0
*
control
Mint KO
Mint2 WT
Mint2 N723S
Mint2 ΔPDZ
rescue
B
wild type background
Mint2 WT
Mint2 N723S
mEPSC frequency (% WT)
mEPSC amplitude (% WT)
20 pA
1 s

Figure. 5.3: Mint2 copy number variant alters synatogenesis.

A, Mint2 copy number variant increases synaptogenesis in wild type hippocampal neurons. Representative images of COS-7 cells expressing NL1-mVenus fusion protein (green) that were cocultured with neurons to induce synapse formation (indicated by synapsin labeling in red). ***B,*** Quantification of synapse formation of Mint2 copy number variant compared to the wild type control.

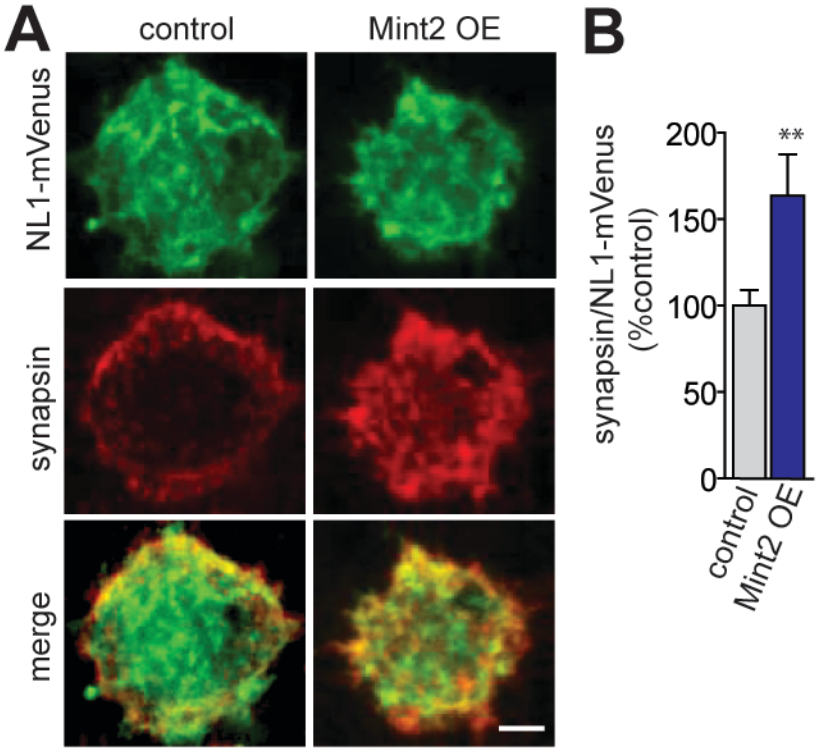


Figure. 5.4: Mint2 copy number variant increases vesicular trafficking and the number of docked vesicles in asymmetric excitatory synapses.

A, Representative electron micrographs of asymmetric excitatory synapses of wild type control or Mint2 overexpressed (Mint2 OE) neurons. ***B,*** Bar graphs showing that Mint2 overexpression significantly increases the vesicles per terminal and the docked vesicles per active zone compared to the wild type control. *p<0,05. Scale bar is 100 nm.

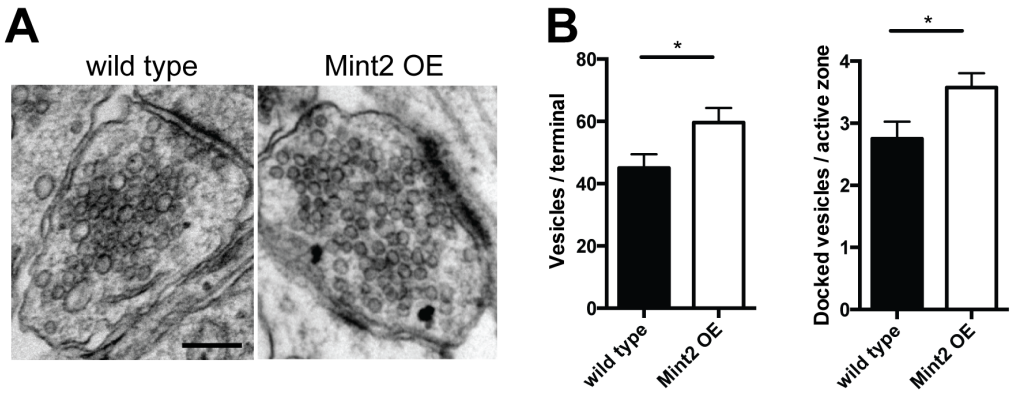


Table. 5.1: Quantitative ultrastructural analysis of the effects of Mint2 copy number variant on excitatory synapses.

Parameter	Wild type	Mint2 OE	p value
Presynaptic terminal area (μm^2)	2.22 ± 0.24	2.65 ± 0.26	p=0.225
Presynaptic terminal circumference (μm)	3.11 ± 0.17	3.45 ± 0.15	p=0.139
Length of postsynaptic density (μm)	0.35 ± 0.02	0.41 ± 0.03	p=0.137
Docked vesicles per active zone	2.75 ± 0.36	3.58 ± 0.23	p=0.024*
Vesicles 150 nm of the active zone	10.35 ± 0.7	10.8 ± 0.80	p=0.703
Vesicles per terminal	45.05 ± 5.5	59.65 ± 4.7	p=0.025*

All data are presented as means ± SEM

n=40 cells /treatment

*Indicates significance.

Table. 5.2: Quantitative ultrastructural analysis of the effects of Mint2 copy number variant on inhibitory synapses.

Parameter	wild type	Mint2 OE	p valu e
Presynaptic terminal area (μm^2)	2.28	1.95	0.35
Presynaptic terminal circumference (μm)	3.53	3.32	0.54
Length of postsynaptic density (μm)	0.36	0.60	0.41
Docked vesicles per active zone	16.91	11.93	0.15
Vesicles 150 nm of the active zone	48.73	49.60	0.93
Vesicles per terminal	22.02	25.51	0.34

All data are presented as means ± SEM
Wild type, n=11; Mint2 OE, n=15.

Table. 5.3: Quantitative ultrastructural analysis of the effects of Mint2 N723S mutant on excitatory synapses.

Parameter	Wild type	N723S	p value
Presynaptic terminal area (μm^2)	2.38 ± 0.19	2.92 ± 0.3	p=0.116
Presynaptic terminal circumference (μm)	3.13 ± 0.15	3.41 ± 0.21	p=0.163
Length of postsynaptic density (μm)	0.31 ± 0.02	0.34 ± 0.02	p=0.251
Docked vesicles per active zone	2.03 ± 0.23	2.04 ± 0.28	p=0.983
Vesicles 150 nm of the active zone	7.5 ± 0.53	7.89 ± 0.48	p=0.598
Vesicles per terminal	49.18 ± 4.4	65.56 ± 7.8	p=0.059
Vesicle density	23.15 ± 2.0	23.41 ± 1.9	p=0.926

All data are presented as means ± SEM
Wild type, n=34; N723S, n=27.

Table. 5.4: Quantitative ultrastructural analysis of the effects of Mint2 N723S mutant on inhibitory synapses.

Parameter	Wild type	N723S	p value
Presynaptic terminal area (μm^2)	2.38 ± 0.33	3.00 ± 0.37	p=0.223
Presynaptic terminal circumference (μm)	3.08 ± 0.27	3.45 ± 0.22	p=0.298
Docked vesicles per active zone	1.43 ± 0.31	1.54 ± 0.35	p=0.816
Vesicles 150 nm of the active zone	6.29 ± 0.57	8.23 ± 0.57	p=0.023*
Vesicles per terminal	39.14 ± 5.7	52.85 ± 5.2	p=0.091
Vesicle density	18.50 ± 2.2	18.75 ± 1.7	p=0.932

All data are presented as means ± SEM
Wild type, n=14; N723S, n=13.
*Indicates significance.

CHAPTER SIX: Thesis research summary and future directions

Thesis research summary

Autism spectrum disorder (ASD) is a neurodevelopmental disorder with strong genetic associations. Mutations in the human *MINT2* gene that encodes for a neuronal adaptor protein have been genetically-linked to autism patients. The *MINT2* gene maps to the distal portion of chromosome 15q13.1, a region that is commonly deleted in Prader-Willi and Angelman syndromes and duplicated in cases of autism, making *MINT2* an attractive candidate gene associated with autism. Copy number variants in the *MINT2* gene have been associated with autism (Babatz *et al.* 2009). Also, seven novel nonsynonymous coding variants in Mint2 in ASD subjects have been identified. Five of the seven unique ASD Mint2 variants were predicted to affect protein function; however, these variants have not been examined functionally.

Mint2 belongs to the Mint family of neuronal proteins that are important for synaptic function. The multi-domain structure and the nature of their interacting partners determine the function of the Mints. Mint2 interacts directly with neurexin-Iα (*NRXN1)*, an ASD gene, as part of a multi-protein complex that acts as a facilitator of neurotransmitter release. The goal of my work is to understand the biology of Mint2 and its potential contribution in the pathogenesis of ASD. I hypothesized that Mint2 is important for neuronal development and that Mint2 copy number and sequence variants contribute to neuronal dysfunctions that are associated with autism. For this project, I came up with the following three aims.

First, I determined whether Mint2 copy number and sequence variant N723S alter neuronal development. I found that while Mint2 copy number variant (a gain-of-function mutation) increases neuronal growth, Mint2 N723S (a loss-of-function mutation) decreases neuronal growth, suggesting that Mint2 is involved in neuronal development.

Second, I found that Mint2 wild type in involved in neurexin I membrane localization and synapse targeting. Also, I found that while Mint2 N723S binds to neurexin normally, it decreases neurexin membrane localization in HEK293T cells and neurexin I synapse targeting in neurons. Together, these data reveal a novel function of Mint2 in neurexin membrane localization and the altered membrane localization resulted from Mint2 N723S could lead to neuronal dysfunction.

Third, I determined whether Mint2 copy number variant and Mint2 N723S alter synapse formation and synaptic function. I found that while Mint2 copy number variant promotes synapse formation, Mint2 N723S decreases synapse formation. Previously publication from the lab has shown that Mint2 copy number variant increases excitatory spontaneous neurotransmission. In contrast, I found that Mint2 N723S decreases excitatory spontaneous neurotransmission. In sum, this study suggests that Mint2 copy number and sequence variant N723S disrupt synapse formation and synaptic transmission that are associated with ASD pathogenesis. The validation and mechanistic role of new genes such as *MINT2* associated with ASD will directly lead to a better understanding of the molecular pathways critical for diagnosis of ASD and may improve prognosis and lay the foundation for novel therapeutic approaches.

In this thesis, I have examined both Mint2 copy number and sequence variant Mint2 N723S in neuronal development (Chapter 3) and neuronal function (Chapter 4 and 5). Copy number variants are the duplication or deletion of DNA, which leads to more or less genetic material, respectively. In this study, I have examined both types of copy number variants in Mint2. First, Mint2 copy number variant (duplication of genetic material) is mimicked by Mint2 overexpression in wild type neurons. Second, Mint2 copy number variant (deletion of genetic material) is mimicked by Mint2 knockout in Mint-deficient neurons. All, I have examined the effect of Mint2 sequence variant N723S in Mint2-deficient neurons. It is important to note that Mint2 N723S behaves like a Mint2 knockout in functional neuronal experiments, including neuronal development (Chapter 3), synaptogenesis (Chapter 5), and neurotransmission (Chapter 5). In summary, Mint2 N723S and Mint2 knockout are loss-of-function mutations whereas Mint2 copy number variant (Mint2 overexpression) is a gain-of-function mutation, and both of them can contribute to deficits in neuronal development and synaptic function.

Future directions

Identify Mint2-neurexin trafficking complex

This thesis has covered an extensive array of *in vitro* studies in both HEK293T cells and primary mouse neurons with techniques ranging from biochemistry, cell biology, live cell imaging, and electrophysiology. Many of these techniques are standard and widely used in the synaptic field. In addition to what has been done, few interesting questions still required further investigation.

First, it would be interesting to decipher the Mint2-neurexin trafficking complex. Previously it was shown that the first PDZ domain of Mint1 interacts directly with kinesin superfamily motor protein KIF17 to traffic NMDA receptor-containing vesicles in the dendrites (Setou *et al.* 2000). Since the PDZ domains are highly conserved within Mints, Mint2 could interact with KIF17 in principle. To determine whether Mint2 interacts with KIF17 directly, I could co-immunoprecipitate Mint2 and KIF17 in HEK293T cells and in wild type neurons. For HEK293T cells, I could immunoprecipitate for Mint2 and immunoblot for KIF17. For wild type neurons, I could immunoprecipitate for Mint2 and immunoblot for KIF17 and neurexin, which would determine whether these proteins are associated together.

One can also use GST pull down as an alternative approach to study Mint2 and KIF17 interaction in both HEK293T cells and wild type neurons. For this experiment, a bacterial expression GST fusion protein that expresses the cytoplasmic tail of neurexin I to test whether neurexin could pull down Mint2 and KIF17.

In addition to the biochemical analyses described above, I could perform immunocytochemistry experiments to validate Mint2, neurexin, and KIF17 interaction in neurons. Since there is no neurexin antibody available for immunostaining, mCherry-neurexin plasmid must be transfected into neurons. The endogenous Mint2 and KIF17 can be labeled with commercially available antibodies to determine whether all three proteins co-localized together, which would suggest interaction.

Lastly, one could investigate the developmental expression of KIF17, Mint2, and neurexin in brain lysate. Brain lysates from wild type pups are immunoblotted with antibodies against endogenous KIF17, Mint2, and neurexin to determine whether they are showing a similar temporal expression during development. Co-expression would indicate co-regulation.

Mint1 was identified as the receptor for KIF17 by yeast two-hybrid screening (Setou *et al.* 2000). It is important to keep in mind that KIF17 may not be the motor protein that is involved in Mint2-mediated neurexin trafficking. There is a fundamental difference between Mints 1 and 2 vesicular transport. For Mint1, Kif17 is involved in the transport (anterograde) of NMDA receptor subunit in the dendrites. For Mint2, I focused on neurexin transport (anterograde) in the axon. Because of the difference in location (dendrite vs. axon), the same motor protein may not apply. If all the above experiments failed, one should seek other methods (e.g., proximity ligation assay or chemical crosslinking with co-immunoprecipitation) to identify potential protein interactions in neurons.

Live cell imaging of GFP-Mint2 and mCherry-Neurexin

When I first found that Mint2 could be involved in neurexin membrane trafficking, the initial goal was to visualize Mint2 and neurexin interaction in live neurons. However, I was not able to visualize the mCherry signals in live cells due to the limitation of our microscope. For this study, I bypassed the limitation by amplifying the mCherry signals with a rabbit anti-mCherry antibody in fixed neurons. However, it

would be informative to visualize Mint2 and neurexin trafficking in live neurons if the technology allows.

Decipher the function of Mint2 in Golgi

The synaptic functions of Mint2 have been well studied in the literature. However, Mint2 is predominately localized in the neuronal Golgi (Biederer *et al.* 2002)—also see Fig. 4.6. It is speculated that Mint2 is involved in vesicle trafficking (Biederer *et al.* 2002). However, the exact function of Mint2 in the Golgi is not clear and requires further investigation, which could reveal a novel function. Also, the two PDZ domains clearly are essential for Mint2 Golgi targeting. It would be interesting to determine their function in Golgi targeting. Mint3 also has two PDZ domains like Mints 1 and 2. Mint3 is ubiquitously expressed throughout the body. Therefore, it is possible to learn about the Golgi function by using Mint3 as a tool.

Mint2 dimer

At the present moment, it is not clear whether Mint2 is monomeric, dimeric, or multimeric. Based on the effects of Mint2 N723S heterozygous mutant on axon development, synapse formation, and neurotransmission, I speculated that Mint2 N723S could be a dominant negative mutation, which strongly suggests that Mint2 proteins can dimerize. Mint2 N723S binds to Mint2 wild type and inhibits its function. To test this hypothesis, co-immunoprecipitation experiment of Mint2 wild type and Mint2 N723S

was performed to determine the potential interaction between them. However, co-immunoprecipitation does not show direct interaction, as there could be other mediators involved. I also tried to visualize Mint2 dimer with native gel electrophoresis. Although a 260 kDa Mint2 band (approximately the size of Mint2 dimer) was visible, the Mint2 monomeric band was not visible (maybe due to the small abundance of Mint2 monomers). The data would be more convincing if both Mint2 dimeric and monomeric bands are visible in the native gel.

Generate and characterize Mint2 N723S knock-in mice

While using Mint conditional knockout mice (Ho *et al.* 2006, Ho *et al.* 2008) to study the effects of Mint2 N723S mutation is a simple and an elegant strategy, it is not sufficient to represent the physiological condition of the patient. Moreover, experiments with neuronal cultures only allow *in vitro* studies. To further investigate the effects of Mint2 N723S *in vivo*, Mint2 N723S knock-in mice should be generated as a tool. There are myriad of experiments that could be done to characterize Mint2 N723S knock-in mice. First, one could examine the synaptic transmission from cultured brain slices to monitor spontaneous miniature postsynaptic currents (minis) and evoked synaptic transmission. Second, one could analyze the morphology of the synaptic structure to determine the effects of Mint2 N723S. Third, one could perform quantitative protein analysis of neuronal proteins by Western blotting and imaging. Lastly, since deficit in social interaction is one of the core criteria for autism diagnosis, it would be important to

test whether Mint2 N723S knock-in mice display similar phenotypes that are associated with autism.

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APPENDIX

A1.1 Mint2 ASD mutants do not alter Mint2 cellular localization

Although Mint2 ASD mutations do not alter Mint2 protein expression in Western blotting analysis (Fig. A1.1), Mint2 ASD mutants may still be mislocalized in cells without any expression deficit. To investigate this possibility, I next determined whether Mint2 ASD mutants alter Mint2 localization. HEK293T cells were transfected with GFP-tagged Mint2 wild type, Mint2 R4Q, Mint2 T660M, or Mint2 N723S on coverslips. These mutants were selected based on their differential binding to APP (Fig. 3.2B-C). 48 h after transfection, coverslips were fixed, mount, and imaged. The data showed that Mint2 wild type has an even cytosolic localization (Fig. A1.1). There was no obvious difference between the mutations compared to the wild type, suggesting that they do not alter Mint2 localization in HEK293T cells. It is important to note that some phenotypes may not be visible in HEK293T cells because these cells are not neuronal and lacking other neuronal proteins that are essential for Mint2 function. For example, HEK293T cells transfected with Mint2 wild type alone does not show Golgi localization. In contrast, primary neurons infected with Mint2 wild type lentivirus does show a Golgi localization.

A1.2 Mint2 ASD mutants do not alter co-localization with APP

Based on co-immunoprecipitation experiments, three Mint2 ASD mutants (R4Q, T660M, and N723S) altered binding to APP (Fig. 3.2B-C). To determine whether altered

interaction also leads to altered APP localization, I examined APP and Mint2 co-localization in HEK293T cells. APP was transfected together with GFP-tagged Mint2 wild type, Mint2 R4Q, Mint2 T660M, or Mint2 N723S. 48 h after transfection, coverslips were fixed and stained for APP. The data revealed that Mint2 wild type and APP co-localize in vacuoles (Fig. A1.2), which is different from Mint2 wild type transfection alone (Fig. A1.1). However, none of the ASD mutants displayed any obvious difference compared to the wild type.

A1.3 Mint2 and APP-containing vacuoles are localized to lysosome and autophagosome

To further characterize the identity of Mint2 and APP-containing vacuoles, APP was transfected together with GFP-Mint2 wild type and stained for various organelle markers. The data revealed that Mint2 and APP-containing vacuoles are localized to either lysosome or autophagosome (Fig. A1.3).

A1.4 Mint2 form tripartite complex with APP and Munc18-1

A previous study found that Mint2, APP, and Munc18-1 form a tripartite complex in both co-immunoprecipitation and Bimolecular Fluorescence Complementation (BIFC) assays (Weyer *et al.* 2011). To reproduce this finding, I transfected GFP-Mint2 wild type, APP, and Munc18-1 (in equal ratio) into HEK293T cells. 48 h after transfection, cells were labeled for APP and Munc18-1. The data revealed that Mint2, APP, and Munc18-1 co-localized into vacuoles (Fig. A1.4).

Figure A1.1: Mint2 ASD mutants do not alter Mint2 cellular localization in HEK293T cells.

Representative images of HEK293T cells transfected with GFP-Mint2 wild type (WT), Mint2 R4Q, Mint2 T660M, or Mint2 N723S.

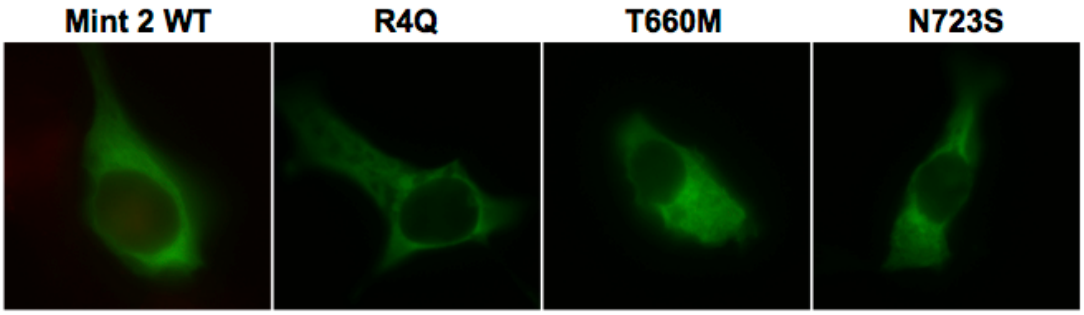


Figure A1.2: Mint2 ASD mutants do not alter colocalization with APP in HEK293T cells.

Representative images of HEK293T cells transfected with APP with GFP-Mint2 wild type (WT), Mint2 R4Q, Mint2 T660M, or Mint2 N723.

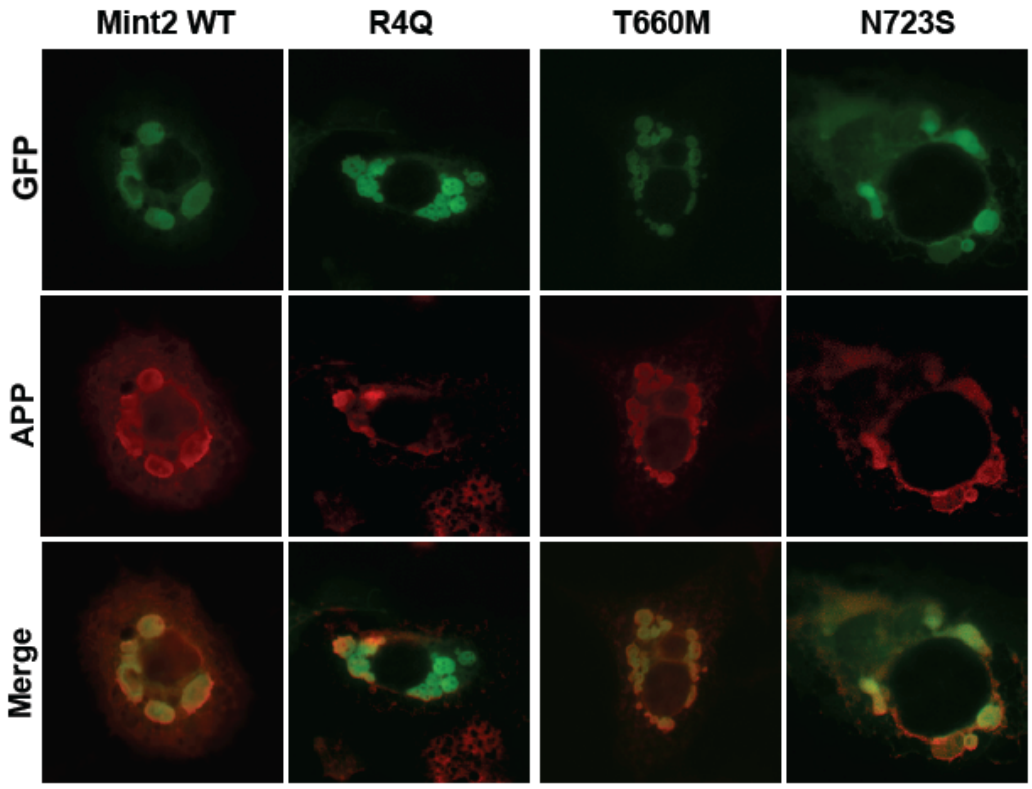


Figure A1.3: Mint2 and APP-containing vacuoles are localized to autophagosome and lysosome in HEK293T cells.

Representative images of HEK293T cells transfected with GFP-Mint2 wild type (WT) and APP. Cells were fixed and stained with antibodies against LAMP (an autophagosome marker) or LC3 (a lysosome marker). Note that the APP staining is not shown here.

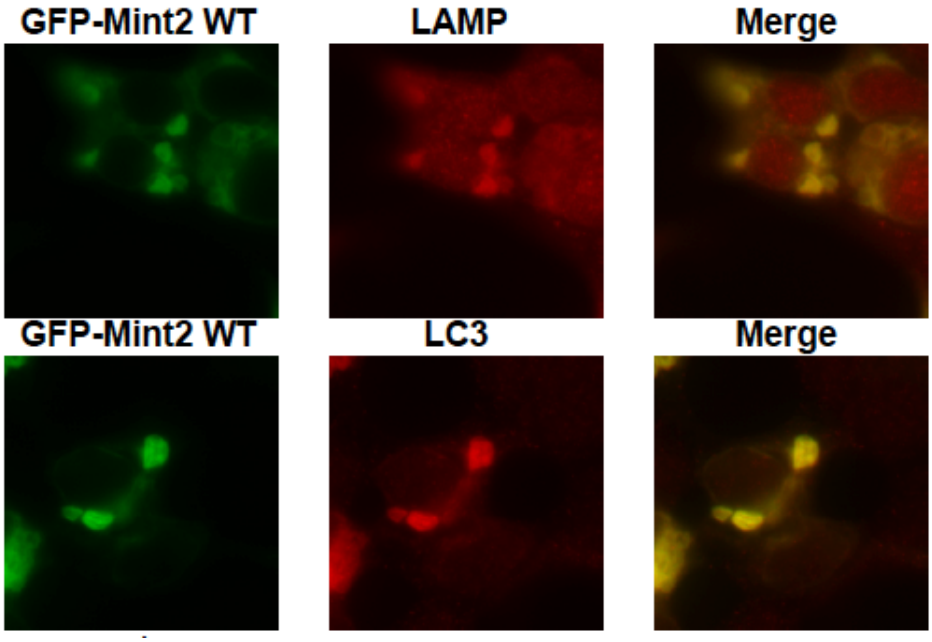
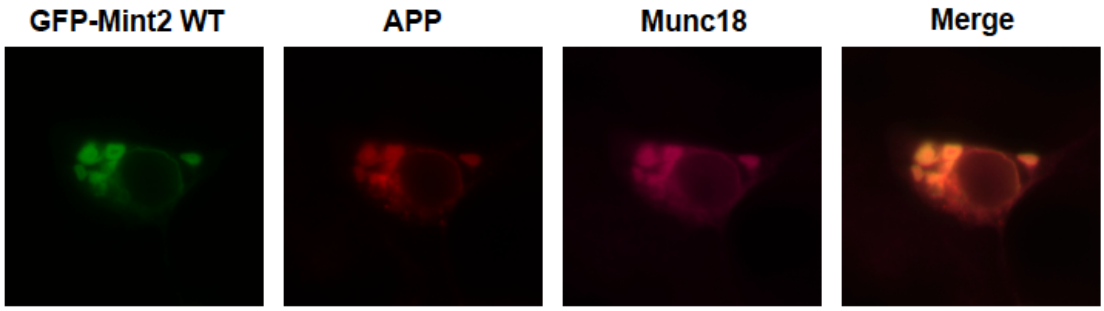


Figure A1.4: Mint2 forms tripartite complex with APP and Munc18-1 in HEK293T cells.

Representative images of HEK293T cells transfected with GFP-Mint2 wild type (WT), APP (in red), and Munc18 (in pink). Cells were fixed and stained with antibodies against APP and Munc18.



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